PATENT
Attorney Docket No. 10142.0001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
Donald GULLBERG	) Group Art Unit: 1644
Application No.: 09/980,403	) Examiner: Haddad, Maher M.
Filed: April 15, 2002	)
For: AN INTEGRIN HETERODIMER AND AN ALPHA SUBUNIT THEREOF	) Confirmation No.: 3147

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Donald Gullberg, do hereby make the following declaration:

1. I am a Professor of Biomedicine at the University of Bergen, Norway.

2. My *curriculum vitae* is provided as Appendix 1.

3. I am the sole inventor of the invention disclosed in US Patent Application No. 09/980,403, which relates to the human integrin alpha-11 subunit and its use.

4. I have reviewed the Office communication dated July 18, 2007, issued in connection with US Patent Application No. 09/980,403, including the references cited therein.

5. I am the same Donald Gullberg who co-authored the following references cited by the Examiner on page 3 of the Office communication:

Gullberg et al., *Dev. Dyn.* 204:57-65 (1995)

Velling et al., *J. Biol. Chem.* 274:25735-42 (1999)

6. In the following paragraphs I state my opinion as to certain assertions made by the Examiner in the communication dated July 18, 2007.

7. The Examiner asserts that "Gullberg et al teach an isolated integrin subunit αmt obtained from G6 myoblasts and myotubes." Office communication, page 3. I disagree with this assertion because Gullberg et al did not isolate integrin αmt, or any other new integrin molecule, from G6 myoblasts and myotubes. Gullberg et al only identified a biochemical entity that was proposed to be a new integrin α chain based on certain biophysical properties that were reminiscent of known integrin α chains and the lack of immunological cross-reactivity with some of the known integrin α chains. The mentioned biophysical properties were (1) the interaction with integrin subunit β1 as observed by co-immunoprecipitation and (2) the size in polyacrylamide gels. See Gullberg et al., page 58, left column, last paragraph, and page 59, first paragraph.

Hence, the integrin αmt of Gullberg et al is a biochemical entity that was characterized almost exclusively by immunological methods. This entity was not isolated or purified. Gullberg et al does not contain a single experiment that was designed to isolate or purify integrin αmt from the cell extracts.

8. The Examiner asserts that the claimed invention differs from the reference teachings only by the recitation of different domains of integrin subunit α11 and of amino acids of SEQ ID NO:2 comprised in the domains. Office communication, page 3. I disagree with this assertion because Gullberg et al did not disclose any new integrin

molecule that is identifiable by a specific amino acid sequence. Whether the new integrin αmt proposed by Gullberg et al is related, or even identical, to the integrin subunit α11 or SEQ ID NO:2 disclosed in application 09/980,403 remains unknown to this day. Gullberg et al did not disclose any amino acid sequence that would allow such a conclusion. Hence, the claimed invention differs from the reference teachings not only by disclosing specific domains of integrin subunit α11, but also by disclosing integrin subunit α11 itself. The statement by Velling et al. that the integrin αmt of Gullberg et al correlates to integrin subunit α11 is not based on any sequence information but solely on the comparison of a very limited number of biophysical properties (see my Declaration dated November 10, 2005, and Teet Velling's Declaration dated November 15, 2005). In fact, Gullberg et al left open the relation between the biochemical entity termed integrin αmt and integrin α chains that were not addressed in the study, alternative splice variants of integrin α chains, and any unrelated integrin subunit β1 interaction partners.

9. The Examiner asserts that based on the combined teachings of the cited references one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Office communication, page 5. I disagree with this assertion for the following reasons.

(a) One of ordinary skill in the art at the time of the invention knew about the enormous complexity of the integrin family, in particular with respect to its large number of related genes and proteins, its complex expression patterns, and its complex regulation. Eleven (11) integrin α chains that associate with integrin β1 had been identified and characterized. Furthermore, it was generally accepted that the

identification of integrin proteins was complicated by the occurence of alternative splicing of integrin chains.[1] Moreover, it was well established that integrins are expressed in a cell type-dependent, differentiation state-dependent, and/or species-specific fashion.[2]

My coworkers and I found this also to be true for the proposed new integrin αmt. Gullberg et al, abstract; page 58, paragraph 3; and Figure 1. Finally, it was known that the activation, posttranslational modification, and even sequence of various integrins depend on cell type and differentiation state.[3]

(b) In my opinion, one of ordinary skill in the art at the time of the invention would have taken one of three approaches to clone the cDNA of an unknown integrin subunit, such as the integrin subunit αmt. In fact, all integrin subunits identified in the period between 1987 and 1996 were cloned by one of these three approaches.[4]

A first approach would have been to affinity purify the integrin subunit, followed by aminoterminal sequencing of the isolated integrin protein, and cDNA library screening using a degenerate DNA probe designed based on the obtained

---

[1] See, e.g., Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34, at page 1724. This reference is provided as Appendix 2.

[2] See, e.g., Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34; Erle et al., J Biol Chem. 1991 Jun 15;266(17):11009-16; Humphries et al., Ciba Found Symp. 1995;189:177-91; discussion 191-9; Shaw and Mercurio, Mol Biol Cell. 1994 Jun;5(6):679-90; Djaffar et al., Biochem J. 1994 May 15;300 (Pt 1):69-74; Collo et al., J Biol Chem. 1993 Sep 5;268(25):19019-24; Song et al., J Cell Sci. 1993 Dec;106 (Pt 4):1139-52. These references are provided as Appendix 3.

[3] See, e.g., Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34, at abstract and pages 1723-4.

[4] See, e.g., Wayner and Carter, J Cell Biol. 1987 Oct;105(4):1873-84; Hogervorst et al., EMBO J. 1990 Mar;9(3):765-70; Sheppard et al., J Biol Chem. 1990 Jul 15;265(20):11502-7; Erle et al., Am J Respir Cell Mol Biol. 1991 Aug;5(2):170-7; Hogervorst et al., Eur J Biochem. 1991 Jul 15;199(2):425-33; Palmer et al., J Cell Biol. 1993 Dec;123(5):1289-97; Van der Vieren et al., Immunity. 1995 Dec;3(6):683-90; Wong et al., Gene. 1996 Jun 1;171(2):291-4. These references are provided as Appendix 4.

aminoterminal amino acid sequence. For affinity purification, antibodies would have been used that were raised to cell surface proteins and selected based on their ability to block adhesion of cells to extracellular matrix proteins. Alternatively, affinity purification would have been attempted via the interaction of the integrin subunit with another, known integrin subunit (such as integrin β1 in case of integrin αmt).

A second approach would have been to use the above antibodies for the screening of a cDNA expression library.

A third approach would have been to screen a cDNA library with a DNA probe that was either derived from a PCR amplification product or by *in silico* design based on regions of homology between already known integrin subunits. The PCR amplification product would have been obtained using degenerate primers, designed based on regions of homology between already known integrin chains, and reverse-transcribed mRNA from the known cellular source as template.

In each of these three approaches the source material (i.e. the cells to which antibodies are raised; the cells from which the integrin subunit is affinity purified; the cells from which cDNA or expression libraries are constructed; and the cells from which mRNA is isolated) would have been the same type of cells in which the integrin subunit had been identified. Hence, in case of integrin subunit αmt the source material would have been human myotube cells, and more specifically G6 myotube cells. See Gullberg et al., Figure 1. The reason for this is that one of ordinary skill in the art would have been very reluctant to change cell source because the art at the time had taught that integrins are expressed in a cell type-dependent, differentiation state-dependent,

and/or species-specific fashion and that alternative splice variants of various other integrin alpha and beta chains further complicate the integrin expression pattern.

We tried to clone the proposed integrin αmt by each of these three approaches, but we failed. For example, we attempted to obtain monoclonal antibodies against integrin αmt for the purpose of cloning integrin αmt by affinity purification or screening of an expression library. However, immunization of mice with G6 myotubes failed to yield any antibodies against integrin αmt. We also attempted to affinity purify integrin αmt using monoclonal antibodies against the interaction partner integrin β1. However, despite collection of large numbers of cultured myotubes for immunoaffinity chromatography this approach also failed. Finally, we also attempted to amplify the cDNA of integrin αmt by PCR using mRNA from myotubes as template and degenerate primers based on conserved regions of known integrin α chains. However, such PCR reactions amplified several other, known integrin α chains, but failed to amplify integrin αmt. For discussion of our unsuccessful attempts to clone integrin αmt by each of the above approaches see also Gullberg et al., page 61, paragraph 2; Velling et al., page 25737, first paragraph of Results and Discussion; and the specification of the instant application at page 19, lines 24-28.

Others are likely to have tried and failed as well. For example, evidence for the existence of a separate, unknown collagen receptor on fibroblasts had been reported as early as 1991.[5] One report provided evidence that fibroblasts contain a collagen receptor comprising integrin β1 and an unknown integrin α chain that mediates reorganization and contraction of three-dimensional collagen I gels by cultured

fibroblasts.[6] Another report provided evidence that fibroblasts contain a collagen receptor comprising integrin β1 and an unknown integrin α chain that mediates cell spreading on collagen I substrata.[7] In both scenarios the unknown integrin α chain is likely to have been integrin αmt or integrin α11. However, none of the involved researchers identified and isolated the unknown integrin entity, despite the fact that these researchers have been significant players in the integrin field since around 1991[8] and there was an intense interest in the field in identifying new integrin molecules due to the recognized biological and medical importance of integrins. I am convinced that the researchers' failure to identify and isolate the unknown integrin entity is not a coincidence, but is due to the seemingly insurmountable difficulties in cloning this integrin by any of the approaches that one of ordinary skill in the art at the time of the invention would have used.

(c) There was intense competition among many laboratories at the time of the invention to beat each other in cloning any new integrin molecule, because the importance of integrins for human disease had been well recognized.[9] Yet, despite

---

[5] See, e.g., Klein et al., J Cell Biol. 1991 Dec;115(5):1427-36; Gardner et al., Dev Biol. 1996 May 1;175(2):301-13. These references are provided as Appendix 5.

[6] Klein et al., J Cell Biol. 1991 Dec;115(5):1427-36.

[7] Gardner et al., Dev Biol. 1996 May 1;175(2):301-13.

[8] Listings of integrin-related publications of two of the researchers (Humphrey Gardner and Thomas Krieg) are provided as examples in Appendix 6.

[9] See Expert Declaration by Dr. Staffan Johansson, page 2; see also, e.g., Van der Vieren et al., Immunity. 1995 Dec;3(6):683-90 ("Despite extensive research in this field, only three members of this integrin subfamily have been described: CDIIa/CD18 (LFA-I), CDIIb/CD18 (Mac-I), and CDIIc/CD18 (pI50,95). We have identified a cDNA encoding a fourth α chain, αd, that associates with CD18." (emphasis added)); Kumar, Oncogene. 1998 Sep 17;17:1365-73 ("The signals from integrin receptors are integrated from those originating from growth factor receptors in order to organize the cytoskeleton, stimulate cell proliferation and rescue cells from matrix detachment-induced programmed cell death. These functions are critical in the regulation

these intense efforts, and despite the fact that new integrin chains were cloned by the above described approaches at a rapid pace in the early 1990's (see footnote 4), no one was able to clone integrin subunit αmt in the years between the publication of integrin αmt in 1995 and the publication of integrin α11 in 1999. In fact, no new α chain could be cloned in the years between 1996 and 1998. Thus, it was generally assumed in the integrin research community during this period that all existing α chains had already been identified. Several publications of the cloning of "integrin-like" proteins -- which turned out not to be true integrin proteins -- further enhanced the belief that all true integrin α chains had been identified and fostered a general skepticism towards any report of a newly identified integrin protein.[10]

(d) Because of the complexity of the integrin family (see 9.(a)) one of ordinary skill in the art would have most likely isolated cDNAs of known integrin α chains or of alternative splice variants thereof, rather than of any new integrin α chain, by using any of the approaches described above. This was born out by our finding that PCR with degenerated primers amplified multiple different integrin α chains, including at least integrin α1, α3, α4, α5, α6, α7, and αv, from G6 myotubes but failed to amplify any new integrin α chain. Gullberg et al, page 58, paragraph 3, and page 59, paragraph 2. Integrin α7 which is expressed in G6 myotubes is a prime example of the complicated expression pattern of alternatively spliced products derived from the same gene.[11]

---

of multiple processes such as tissue development, inflammation, angiogenesis, tumor cell growth and metastasis and programmed cell death." (emphasis added)). These references are provided as Appendix 7.

[10] See, e.g., Gale et al., Proc Natl Acad Sci USA. 1996 Jan 9;93(1):357-61; Berg et al., Genomics. 1999 Mar 1;56(2):169-78; Laval et al., Biochim Biophys Acta. 1999 Nov 16;1435(1-2):61-70. These references are provided as Appendix 8.

[11] See, e.g., Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34.

(e) Because of the complexity of the integrin family in general (see 9.(a)) and the differentiation state-dependent, selective expression of integrin αmt in myotubes in particular (see Gullberg et al.), one of ordinary skill in the art would not have been motivated to try a different source material in attempts to clone integrin αmt. Clearly, the art at the time of the invention advised against changing the source material when trying to clone an integrin molecule that had been identified by biochemical means in a specific cell type and in a specific developmental or differentiation stage (see footnote 2). It was understood by one of ordinary skill in the art that changing the source material would dramatically reduce the expectation of success.

10. The Examiner asserted that it "would have been obvious to one of ordinary skill in the art at the time of the invention was made to determine the amino acid sequence αmt subunit taught by Gullberg et al using the genetic engineering techniques as taught by Albert et al." Office Action, page 4. I disagree for the following reasons.

(a) The techniques referred to by the Examiner require that a protein is purified to homogeneity. See Alberts et al, page 174, paragraph 4. Only if a sufficient quantity of pure protein is obtained, the N-terminal amino acid sequence can be determined and a cDNA cloned. See Alberts et al, page 262, paragraph 4. However, we did not show isolation or purification of the biochemical entity termed integrin αmt in Gullberg et al. We did not show data that would allow an assessment of the purity or the abundance of integrin αmt. Gel bands obtained by SDS-PAGE and autoradiography or fluorography of immunoprecipitations from surface labeled or metabolically labeled cells (see Gullberg et al., Figures 1-4) do not allow such an assessment, in particular in

view of the complexity of the integrin family described above (see 9.(a)). Furthermore, we did not disclose any biophysical properties of integrin αmt, except for its size and interaction with integrin β1, that would enable someone skilled in the art to design a successful purification strategy. In addition, the detectability of integrin αmt varies greatly with the differentiation state of G6 cells. See Gullberg et al, page 58 and Figure 1.

(b) Isolation of a protein of interest from a cell requires methods for separating the protein from the other thousands of cellular proteins and for detecting the protein of interest. The detection method must be highly specific, simple, fast, and sensitive enough so that only a small proportion of the available material is consumed at each purification step. In Gullberg et al. we did not provide any guidance on suitable methods for separating integrin αmt from the other thousands of cellular proteins, nor did we provide any guidance on suitable methods for detecting integrin αmt during a successful purification scheme.

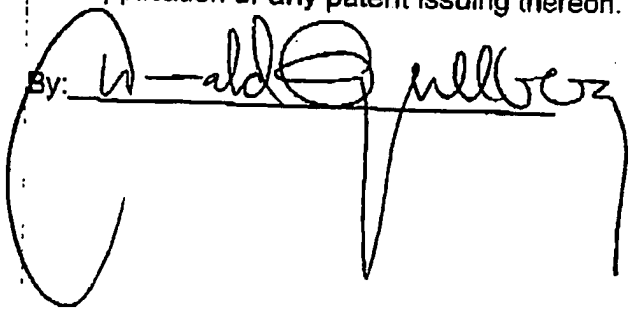
(c) We indicated in Gullberg et al that a cloning strategy as described by Alberts et al would not have a significant chance of success. We expressed our opinion that further biochemical characterization or immunoaffinity purification of integrin αmt will be problematic because of the technical difficulties associated with (1) obtaining sufficient source material and (2) separating integrin αmt from other integrin molecules with similar molecular size. Gullberg et al, page 61, paragraph 2. The seemingly insurmountable technical difficulties of purifying a sufficient quantity of integrin αmt in sufficient purity for successful aminoterminal sequence analysis was born out by the fact that we tried, but failed, to achieve such a purification of integrin αmt.

11. Based on the evidence provided in 7.-10. I believe that one of ordinary skill in the art at the time of the invention -- after considering all the facts and knowledge available -- would not have reasonably expected to be able to clone integrin _mt from a source other than human myotubes, and he/she would have had no good reason to pursue cloning of integrin subunit _mt from uterus tissue (the cDNA of integrin _11 was cloned from an uterus cDNA library; see Velling et al).

12. Based on the evidence provided in 7.-10. I also believe that the prior art, including Gullberg et al, did not put integrin _11 or its domains in the possession of the public.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: January 16, 2008

By: 

APPENDIX 1

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Curriculum Vitae of Donald Gullberg (5 pages)

CURRICULUM VITAE

Name:	Donald Elon Gullberg
Date and place of birth:	1959-03-19 in Arboga, Sweden
Norwegian Personal nr:	190359-19115
Home address:	Rieber Mohns vei 25A, 5231 Paradis, Norway.
University studies:	Major in chemistry, Uppsala university (1980-1984).
Degree:	Dr. Med. Sci. 90-02-21, Dept. of Medical Chemistry, Uppsala Univ.
Postdoctoral studies:	Swedish Natural Science Research council (NFR) postdoc scholarship (June 1990 - November 1992) UCLA, CA. Studies of cell adhesion during muscle development in *Drosophila melanogaster*.
Professor competence:	September 2003, qualified as professor in Medical Biochemistry, Karolinska institute (ranked number one for lectureship in Medical Biochemistry). February 2003, qualified as professor in Medical Physiology (ranked number one for professorship in Physiology at University of Bergen, Norway). November 2002, qualified as professor in Functional Genetics, Lund University (ranked number two for lectureship in functional Genetics).
Present position:	(040101 - present) Professor of Medicine (Physiology), Dept. of Biomedicine, University of Bergen.
Previous positions:	• (990701- 031231) Research scientist, Uppsala University, from 01-09-01- 03-12-31at Medical Biochemistry and Microbiology, Uppsala University. • (990101-990630) "Vikarierande lektor", Department of Cell-& Molecular Biology, Uppsala University. • (930101-981231) "Forskarassistenttjänst" financed by Swedish Medical Research council (MFR), at Dept. of Animal Physiology, UU.
Prizes:	Walter A. Jonsson Prize, Experimental Cell Research and Academic Press, spring -92.
Supervision of PhD students:	Assistant supervisor: Lars Lohikangas, (1995-2000) Main supervisor: Teet Velling (1994-2000, defended thesis 2000-05-18), Carl-Fredrik Tiger (1996-2002, defended thesis 2002-05-16). Current Ph.D students: Ning Lu, University of Bergen spring 2004, Malgorzata Barczyk registered fall 2005, Sergio Carracedo, registered spring 2006

Supervision of postdocs: Wan-Ming Zhang M.D., Ph.D. 00-06-01-02-06-30, Belen Rodriguez-Sanchez, Ph.D. 03-01-12 - 05-01-30, Svetlana Popova M.D, Ph.D. 01-09-01 - present

Commission of trust:

Grant reviewing

• Swedish Research council (2007 - 2010) chairman of Molecular Cell Biology evaluation committee at VR NT-K

• Swedish Research council (2003 - 2006), member of Molecular Cell Biology evaluation committee, 2006 provisional chariman of Molecular Cell Biology evaluation committee at VR NT-K.

• Wellcome trust, Finnish Academy of Sciences, Inflammatory Bowel disease (2000-) grant reviews

Academic expertise:

• Faculty opponent at thesis defense Lisa Rogers Wellcome trust Matrix biology, Manchester UK, 2007-10-05; Alexander Nyström 2007-12-16, Dept. of Cell and Molecular biology, Medical faculty, Lund University; Lachmi Jenndahl 2005-04-22, Medical Biochemistry, Sahlgrenska Akademien, Gothenburg University; Anders Olin 2003-06-11 at Dept. of Cell and Molecular biology, Lund University; Michael Stigson 1996-11-01 at Inst. f. Miljö och Utvecklingsbiologi, Uppsala University.

• Member of thesis committees since 1995-2003 at least 5/year at Uppsala university (Medical/ Natural Science faculties), Karolinska institute, and Lund University.

Recent conferences:

Invited session leader Collagen Gordon conference, Colby Sawyer academy, New Hampshire, USA , July 23-28, 2007

Invited session leader, Swedish-Finnish connective tissue society, Lund, April 13-14, 2007

Invited speaker to Scandinavian Physiology meeting Reykjavik, Iceland August 2006

Invited workshop chairman FECTS meeting in Oulu, Finland, June 2006

Invited Keynote speaker at connective tissue meeting in Munster, Germany Oct. 2005

Editorial boards /Referee for journals:

• Editorial board member Matrix Biology since 2006

• Alliance for Cellular Signaling/Nature (2004 - present) Associate editor on integrins (http://www.signaling-gateway.org/).

• Ad hoc referee for Cancer Res., Cell & Tissue Res. J. Cell Biol., Dev. Dyn., Exp. Cell Res., Eur. J. Cell Biol., Eur. J. Biochem., J. Biol. Chem., J. Cell Sci. , Mol. Med. 1993 - 2002 invited by journal editor as a regular contributor to Trends in Cell Biology "Headline"section

Tasks at UiB

Instituttrådsleder Institutt f. Biomedisin , UiB, 2006-2009

Patents:

Patent application (-99), "use of α11 integrin", patent application initiated by Active Biotech, Lund, currently with Cartela AB, Lund. Patent application (-02), Cartela AB, "use of itga10 and itga11 knockout mice". 2006 partner on patent application with Ming Tsao, "α11 as a stroma marker in lung cancer".

Publications last 5 years

1. Zhu, C.-Q., S. Popova, E.R.S. Brown, D. Barsyte-Lovejoy, R. Navab, W. Shih, M. Li, I. Jurisica, L. Penn, **D. Gullberg**, and M.S. Tsao. 2007. Integrin alpha11 regulates insulin-like growth factor (IGF)-2 expression in fibroblasts and tumorigenicity of human non-small cell lung cancer cells. *Proceedings National Academy of Sciences USA* **104**:11754-11759

2. Popova, S., Barczyk, M., Tiger, C., Beertsen, W.,Zigrino, P., Aszodi, A., Miosge, N., Forsberg, E. and **Gullberg, D.** (2007). α11β1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. *Mol Cell Biol.* **27**:4306-4316

3. Zhang, Z.G., I. Bothe, F. Hirche, M. Zweers, **D. Gullberg**, G. Pfitzer, T. Krieg, B. Eckes, and M. Aumailley. (2006). Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of α2β1 integrin. *J Cell Sci.* **119**:1886-95

4. Zhou, Y.W., D.B. Thomason, **D. Gullberg**, and H.W. Jarrett. (2006). Binding of laminin alpha1-chain LG4-5 domain to alpha-dystroglycan causes tyrosine phosphorylation of syntrophin to initiate Rac1 signaling. *Biochemistry 45:2042-2052*

5. Bystrom, B., I. Virtanen, P. Rousselle, **D. Gullberg,** and F. Pedrosa-Domellof. (2006). Distribution of laminins in the developing human eye. *Invest Ophthalmol Vis Sci.* **47**:777-85.

6. Lu, N., R. Heuchel, M. Barczyk, W.M. Zhang, and **D. Gullberg.** (2006). Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate alpha11 integrin chain expression in mesenchymal cells. *Matrix Biol.* **25**:118-29.

7. Mirtti, T., C. Nylund, J. Lehtonen, H. Hiekkanen, L. Nissinen, M. Kallajoki, K. Alanen, **D. Gullberg**, and J. Heino. (2006). Regulation of prostate cell collagen receptors by malignant transformation. *Int J Cancer.* **118**:889-98.

8. Popova, S Rodriguez-Sanchez, B.,Lidén. Å., Betsholtz, C., van den Bos, T., **Gullberg, D.** (2004) The mesenchymal $\alpha 11\beta 1$ integrin attenuates PDGF-BB stimulated chemotaxis of embryonic fibroblasts on collagens. *Dev. Biol.* **270,** 427-442

9. Zhang, W.-M., Käpyläs, J., Puranen, J.S., Farndale, R., Tiger, C.-F., Pentikäinen, O.T., Johnson, M.S., Heino, J., and **Gullberg, D.** (2003) $\alpha 11\beta 1$ integrin recognizes the GFOGER sequence in interstitial collagens, *J. Biol. Chem.,* **278,** 7270-7278

10. Zhang, W.-M., Bergman,C. , Velling, T., Kusche-Gullberg, M., and **Gullberg, D.** (2002) Analysis of the human integrin $\alpha 11$ gene (ITGA11) and its promoter. *Matrix. Biol.* **21,** 513-523

11. Tiger, C. F., Fougerousse, F., Grundstrom, G., Velling, T., and **Gullberg, D**. (2001) $\alpha 11\beta 1$ integrin is a receptor for interstitial collagens involved in cell migration and collagen reorganization on mesenchymal nonmuscle cells *Dev Biol* **237,** 116-129.

Reveiw articles

12. Gullberg, D (2007) Neuronal pathways leading to the kidney. *Matrix biology* **26,** 407-408

13. Popov□' S., Lundgren-Åkerlund, E., Wiig, H., and **Gullberg, D.** (2007) □□to□□. *Acta Physiologica in* 190:*179-187*

14. Gullberg, D. (2003) Cell biology: the molecules that make muscle *Nature* **424,** 138-140.

15. Gullberg, D. (2002) Yet another liaison between two cell adhesion families *Trends Biochem. Sci* **27,** 602.

16. Gullberg, D. (2002) Importance of ECM remodeling clarified *Trends Cell Biol.* **12,** 110.

17. Gullberg, D. E., and Lundgren-Akerlund, E. (2002) Collagen-binding I domain integrins--what do they do? *Prog Histochem Cytochem* **37,** 3-54

Book chapters and books

18. Gullberg, D, Popova, S.N., and Tiger C-F (2003) Structure and function of $\alpha 11\beta 1$ integrin. In "I domains in integrins" (Ed. **Gullberg, D)**, Medical Intelligence Unit of Bioscience Publishers, LANDES Bioscience, Austin, Texas, pp 67-81

Manuscripts

19. Wolf, K., Barczyk, M. , Rodriguez -Sanchez,B., Bergman, C., Friedl, P. and **Gullberg, D.** (2005). $\alpha 11\beta 1$ integrin dynamics in a three dimensional collagen matrix.*manuscript**

20. Tu, H., Rodriguez-Sanchez, B., Popova, S., Zhang, W-M, **Gullberg, D.**, Pihljaniemi, T. (2005) Type XIII collagen is a high-affinity ligand for $\alpha 11\beta 1$ integrin. *manuscript**

•Manuscripts are available upon request as pdf files (donald.gullberg@biomed.uib.no)

• My publications have been cited more than 2100 times (August 2007) and my current h-index is 25.

APPENDIX 2

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34 (12 pages)

Molecular Biology of the Cell
Vol. 8, 1723–1734, September 1997

The Laminin-binding Activity of the α7 Integrin Receptor Is Defined by Developmentally Regulated Splicing in the Extracellular Domain

Barry L. Ziober,* YaoQi Chen,* and Randall H. Kramer*†‡

*Department of Stomatology and †Department of Anatomy, University of California, San Francisco, California 94143-0512

Submitted May 5, 1997; Accepted June 24, 1997
Monitoring Editor: Mary Beckerle

The expression pattern of the laminin-binding α7β1 integrin is developmentally regulated in skeletal, cardiac, and smooth muscle. The X1/X2 alternative splicing in the extracellular domain of α7 is found in the variable region between conserved α-chain homology repeat domains III and IV, a site implicated in ligand binding. To assess differences in X1/X2 isoform activity, we generated MCF-7 cell lines transfected with α7-X1/X2 cDNAs. Transfectants expressing the α7-X2 variant adhered rapidly to laminin 1, whereas those expressing α7-X1 failed to attach. That α7-X1 exists in an inactive state was established in assays using an activating β1 antibody that induced X1-dependent cell adhesion and spreading. Furthermore, the activation of α7-X1 was cell type specific, and when expressed in HT1080 cells, the integrin was converted into a fully functional receptor capable of promoting adhesion. Thus, the expression of the α7-X1/X2 integrin is a novel mechanism that regulates receptor affinity states in a cell-specific context and may modulate integrin-dependent events during muscle development and repair.

INTRODUCTION

Integrins are members of a large family of cell surface receptors that mediate adhesive interactions with extracellular matrix macromolecules (Hynes, 1992). Numerous studies have indicated that integrins are dynamically regulated, existing in a relatively nonfunctional state in which they are incompetent to bind ligand or in a fully active ligand bound form (Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). Frequently, the ligand-binding regions of integrin subunits are cryptic and only become exposed when the subunit has been activated. A number of factors, originating from outside the cell or from the cell interior, can shift the inactive extracellular conformation of the integrin to an active one. For example, antibodies that stimulate integrin function have been shown to bind the extracellular domain of the β1 subunit (Takada and Puzon, 1993; Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). These activating antibodies bind numerous epitopes, located primarily in the N-terminal region and cysteine-rich region of the β1 subunit, that either induce or stabilize the integrin complex in an active conformation that is competent to bind ligand (Arroyo *et al.*, 1992; Takada and Puzon, 1993; Bazzoni *et al.*, 1995). It is also well established that divalent cations can alter integrin-ligand interactions. For example, Mn^{2+} and Mg^{2+} can promote ligand binding, whereas Ca^{2+} acts as an inhibitor for a number of the integrin complexes (Humphries, 1996; Sanchez-Mateos *et al.*, 1996). Because integrin ligand recognition sites lie at or near the divalent cation binding regions, it has been proposed that divalent cations can directly induce a conformational change that exposes the ligand binding sites (Mould, 1996).

In contrast to external factors such as cations and stimulating antibodies, other effectors can activate integrin subunits intracellularly. For example, phorbol esters, by simulating the protein kinase C pathway, can increase the adhesiveness of several cell types through specific integrins (Shimizu *et al.*, 1990; Diamond and Springer, 1994). Finally, activation of cer-

‡ Corresponding author: Departments of Stomatology and Anatomy, University of California at San Francisco, Box 0512, San Francisco, CA 94143-0512.

tain integrins is dependent on cell-type–specific regulation (Chan and Hemler, 1993) that may be due to posttranslational modifications, variations in the sequences of the α or β subunits, and/or the activation of different signal transduction molecules (Chan and Hemler, 1993; Hughes *et al.*, 1997).

Additional complexity of integrin regulation is exemplified by that fact that integrin subunits can be alternatively spliced. This alternative splicing has been shown to occur not only in a number of mammalian α and β subunits but also in *Drosophila* integrin receptors (Brown *et al.*, 1989; van Kuppevelt *et al.*, 1989; Suzuki and Naitoh, 1990; Cooper *et al.*, 1991; Hogervorst *et al.*, 1991; Tamura, *et al.*, 1991; Languino and Ruoslahti, 1992; Collo *et al.*, 1993; Song *et al.*, 1993; Ziober *et al.*, 1993; Djaffar *et al.*, 1994; Delwel *et al.*, 1995; Meredith *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). Such splicing, which generally takes place in the cytoplasmic and the extracellular domains, can give rise to a number of subunit isoforms with diverse functional properties (Grinblat *et al.*, 1994; Fornaro *et al.*, 1995; Meredith *et al.*, 1995; Tennenbaum *et al.*, 1995; van der Flier *et al.*, 1995; Belkin, *et al.*, 1996; Roote and Zusman, 1996).

A laminin-binding integrin complex designated $\alpha7\beta1$ has recently been identified and shown to be expressed in skeletal, cardiac, and smooth muscle (Kramer *et al.*, 1991; Song *et al.* 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993; Yao *et al.*, 1997). Investigating the function of this receptor has been complicated by the number of alternatively spliced isoforms of the $\alpha7$ subunit (Song *et al.* 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993). In the cytoplasmic domain, two isoforms, A and B, are expressed in a developmentally specific manner in skeletal muscle. Although an $\alpha7$C variant has been reported in rat myoblasts, its role in development is unknown (Song *et al.*, 1993). Alternative splicing, in the extracellular domain of this subunit, also produces two muscle-specific developmentally regulated isoforms, designated X1 and X2 (Ziober, *et al.*, 1993). This extracellular alternative splicing occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site, where it may define ligand specificity, affinity, or even ligand binding competence (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). In this study we transfected $\alpha7$-X1 and $\alpha7$-X2 into MCF-7 cells to characterize the function of each isoform. $\alpha7$-X2 bound laminin readily, but $\alpha7$-X1 bound only when activated by the $\beta1$-activating monoclonal antibody (mAb)[1] TS2/16, indicating that alternative splicing regulates $\alpha7\beta1$ ligand binding competence. Furthermore, we show that activation of this isoform is regulated in a cell-specific manner.

[1] Abbreviations used: mAb, monoclonal antibody; OPG, octyl β-D-glycopyranoside.

MATERIALS AND METHODS

Cell Culture and Materials

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection and maintained in DMEM H-16 with 10% fetal bovine serum. The human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection and maintained in DMEM plus 10% fetal bovine serum. Human plasma fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA). Laminin 1 and the laminin E8 fragment were purified from mouse Engelbreth–Holm–Swarm tumor as described previously (Kramer *et al.*, 1991; Kramer, 1994). Human laminin 5 was kindly provided by Dr. Robert Burgeson (Cutaneous Biology Research Center, Boston, MA). Human type I collagen was obtained from Collagen Biomaterials (Palo Alto, CA).

Antibodies against integrin subunits included the rat anti-human $\beta1$ mAb A2B2 and the rat anti-human $\alpha5$ mAb B2G2, kindly provided by Dr. Caroline Damsky (University of California, San Francisco, CA); mouse anti-human $\alpha2$ mAb VM1, kindly provided by Dr. Vera Morhenn (SRI International, Menlo Park, CA); the $\beta1$-activating antibody TS2/16, kindly provided by Dr. Martin Hemler (Harvard Medical School, Boston, MA), and LM230, an anti-human αv mAb, kindly provided by Dr. Dean Sheppard (San Francisco General Hospital, San Francisco, CA). Anti-human $\alpha3$ mAb P1B5 was purchased from Life Technologies (Gaithersburg, MD); rat anti-human $\alpha6$ mAb GoH3 was purchased from AMAC (Westbrook, ME). The rabbit polyclonal antibody 1211 was prepared in this laboratory against peptide sequences specific to the $\alpha7$B cytoplasmic region (GTIQRSNWGNSQWEGSDAH; Yao *et al.*, 1996b). Rat anti-mouse $\alpha7$ mAbs CA5, CY4, and CY8 were generated in this laboratory as described previously (Yao *et al.*, 1996a). Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and ECL kit were purchased from Amersham (Arlington Heights, IL).

Transfection

The construction of $\alpha7$-X2B cDNA has been described (Yao *et al.*, 1996b). Primers (5′ primer, 5′-CTCGACAGGAAGTGGACCAG-3′; 3′ primer, 5′-TGATCCCAAACATGGAATCAG-3′) that flank the X1/X2 splice site were used to amplify a 430-bp fragment from C2C12 myoblast mRNA by using reverse transcription-coupled polymerase chain reaction (PCR) and Vent polymerase (with proofreading activity, New England Biolabs, Beverly, MA). This fragment was digested with *Esp*I and *Apa*I and ligated into the corresponding sites of the $\alpha7$B cDNA. Positive clones containing the X1 insert were identified by PCR using primers specific for X1 and repeat region IV (5′ primer, 5′-GCCAGGGTGGAGCTCTG-3′; 3′ primer, 5′-CTATCCTTGCGCAGAATGAC-3′). The X1 PCR inserts were confirmed by double-stranded DNA sequencing.

Transfection of MCF-7 cells was performed by the calcium phosphate precipitation method (Mammalian Transfection kit, Stratagene, La Jolla, CA). MCF-7 cells at 30% confluency were transfected with 25–30 μg of DNA/10-cm plate. Cells were selected in growth medium containing 500 μg/ml G418. Individual clones or cell lines were isolated by using cloning rings or by fluorescence-activated cell sorting (FACS). Expression of $\alpha7$ in positive clones was verified by Western blot analysis using polyclonal antibody 1211 (Yao *et al.*, 1996b).

Western Blot

Transfected cell lines and parental cells were solubilized with SDS-solubilization buffer (50 mM Tris, pH 7.5, 0.5% Triton X-100, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM *N*-ethylmaleimide). Equal amounts of protein were separated by

SDS-PAGE on 7.5% gels under nonreducing conditions, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then incubated with α7 polyclonal antibody 1211 followed by goat anti-rabbit IgG-HRP. Migration of the α7 subunit was determined by using an enhanced chemiluminescence (ECL) detection system (Amersham).

Flow Cytometry

After detachment with 2 mM EDTA, single-cell suspensions of 10^6 cells/ml were incubated with optimal concentrations of primary antibodies in wash buffer (2% normal goat serum in phosphate-buffered saline [PBS]) for 1 h on ice. Cells were washed three times and incubated with secondary fluorescein-labeled antibodies for 30 min on ice. After washing three times again, the cells were stained with propidium iodide (1 μg/ml) to identify nonviable cells. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson). Control samples consisted of cells with or without secondary antibody binding. Nonviable cells stained with propidium iodide were eliminated from the analysis. FACS-isolated cell lines were sorted two or three separate times.

Immunoprecipitation of Surface Biotin-labeled Cells

Cells were washed twice with PBS and then labeled with sulfosuccinimidyl-6(biotinamide)-hexanoate (NHS-LC)-biotin (Pierce, Rockford, IL), at 1 mg/ml, in PBS at 4°C for 90 min. To stop labeling, cells were washed twice with 50 mM glycine blocking buffer followed by a 10-min incubation in same buffer. Cells were lysed in PBS with 0.1 M Tris, pH 7.5, 2% Nonidet P-40, 2 mM PMSF, and 1 mM *N*-ethylmaleimide and then precleared with protein A beads. Cell lysates were mixed by rotation with primary antibody and protein A beads for ≥3 h. Beads were washed with 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.1% bovine serum albumin (BSA) three times and heated at 100°C in SDS sample buffer for 5 min. The samples were reduced with 2-mercaptoethanol, separated by SDS-PAGE on 7.5% gels, and detected by streptavidin-HRP and ECL.

Cell Adhesion Assay

Microtiter plates (96-well Immulon plates, Dynatech, Chantilly, VA) were coated with matrix proteins or antibodies at the indicated concentrations in PBS for 1 h at 37°C in a humidified atmosphere. Plates were washed with PBS and incubated with medium containing 0.1% BSA for 60 min in a CO_2 incubator to block nonspecific adhesion. Single-cell suspensions were prepared in DMEM containing 0.1% BSA at 4×10^5 cells/ml, added in triplicate to 96-well plates, and then incubated for 30–60 min at 37°C. Nonadherent cells were removed by shaking on a titer-plate shaker (Lab-Line Instruments, Melrose Park, IL) and washed with PBS. Cells were fixed with 1% formaldehyde, stained with 1% crystal violet, and solubilized in 2% SDS; absorbance was then read at 562 nm. Cells bound to collagen (10 μg/ml) on a separate plate were used to represent 100% attachment. Background cell adhesion to 1% BSA-coated wells was subtracted from all readings. The effect of specific blocking antibodies was tested by preincubating the cells with the indicated dilutions of purified antibodies on ice for 30 min prior to the assay.

Laminin-Sepharose Affinity Chromatography

Binding of the α7 integrin on laminin E8 was performed as detailed by Kramer (1994). The E8 fragment of laminin 1 was coupled to CNBr-activated Sepharose to yield 0.5–1.0 mg/ml of packed gel. Cells were washed twice with PBS and then labeled with NHS-LC-biotin as described above. Labeled cells were then extracted with buffer containing 200 mM octyl β-D-glycopyranoside (OPG), in 50 mM Tris-HCl, pH 7.4, 1 mM $MnSO_4$, and 1 mM PMSF for 30–60 min at 4°C. The cell lysate was centrifuged first at $2000 \times g$ to remove nuclei and then at $20,000 \times g$. One milliliter of supernatant was mixed slowly by inversion with ~300 μl of packed E8-Sepharose overnight. After application of the cell extracts, the column was carefully washed with three to five column volumes of OPG buffer. Finally, the bound α7β1 complex was eluted with 10 mM EDTA followed by stabilization with excess Mg^{2+}. Fractions containing eluted integrin subunits were subjected to immunoprecipitation with the appropriate antibodies, separated in an SDS-PAGE gel under reduced and nonreduced conditions, and detected by ECL.

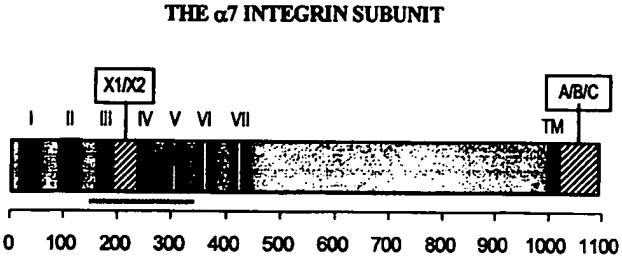


Figure 1. Structure of the α7 subunit. Diagram shows the relative positions of the identified splice sites in the extracellular domain (X1 and X2) and cytoplasmic domain (A/B/C). The seven homology repeat domains (I–VII solid boxes), the divalent cation-binding sites (open slots), the putative ligand-binding site (dotted line, residues 140–350), and the transmembrane domain (TM) are indicated.

RESULTS

Generation of α7-X1- and α7-X2-expressing Cell Lines

We previously reported that the α7 integrin subunit is a laminin-binding integrin that is alternatively spliced in the extracellular domain, producing two isoforms, designated X1 and X2 (Ziober *et al.*, 1993). This alternative splicing occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Figure 1), which could define ligand specificity, affinity, or integrin activation state. To explore for functional differences between the alternatively spliced extracellular isoforms of α7, we constructed expression vectors for α7-X1 and α7-X2. The cDNAs were then stably transfected into human MCF-7 carcinoma cells, which normally adhere poorly to laminin (Yao *et al.*, 1996b).

After selection with G418, several α7-X1- and α7-X2-expressing cell lines were isolated either by FACS sorting or by cloning rings. Two α7-X1-expressing cell lines, X1-A and X1-S3, and two α7-X2 cell lines, X2-A and X2–6, were analyzed further. FACS analysis, using the anti-α7 CY-8 mAb, verified that all cell lines expressed equivalent levels of the integrin (Table 1). In addition, Western blotting confirmed that all α7-X1 and α7-X2 MCF-7 cell lines expressed similar levels of the α7 subunit, whereas the parental MCF-7 cells were negative for the receptor (Figure 2A). Interestingly, α7-X1 displayed a slight decrease in gel mobility when compared with that of α7-X2. Molecular mass calcu-

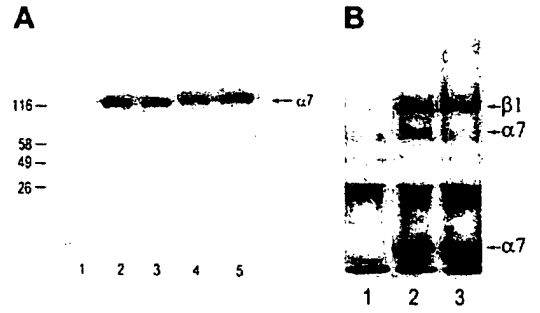


Figure 2. Expression of X1 and X2 transfectants in MCF-7 cells. MCF-7 cells were transfected with pRc/CMV-α7-X1 and pRc/CMV-α7-X2. (A) Equal quantities of cellular lysates from parental MCF-7 (lane 1), X2–6 (lane 2), X2-A (lane 3), X1-A (lane 4), and X1-S3 (lane 5) cells were processed for SDS-PAGE in a 7.5% polyacrylamide gel. After transfer, the nitrocellulose membrane was probed with anti-α7 (1211) antiserum and the position of the α7 subunit was determined by using ECL. (B) Surface-biotinylated parental MCF-7 cells (lane 1) and X1 (X1-A, lane 2) and X2 (X2-A, lane 3) transfectants were immunoprecipitated with anti-α7 polyclonal antibody 1211. Immunoprecipitates were separated by SDS-PAGE on 7.5% gels under reducing conditions to resolve the α7 subunit from comigrating β1 and transferred to nitrocellulose membranes. When reduced, the α7 subunit separates into its heavy and light chains (Yao *et al.*, 1996b). The heavy α7 chain is not strongly biotinylated (Yao *et al.*, 1996b). Proteins were visualized by incubation with streptavidin-HRP and then detected by ECL. The positions of the α7 and β1 subunits are indicated.

lations indicated that this size difference cannot be due to the four additional amino acids encoded by exon X1 as compared with exon X2 (Ziober *et al.*, 1993). This variation in electrophoretic mobility reflects an ~3300-kDa change in molecular mass between α7-X1 and α7-X2, suggesting that there is a conformational difference between the two isoforms. When samples were subjected to SDS-PAGE under reducing conditions, no differences in electrophoretic motility were detected, suggesting that the two α7-X1 and α7-X2 isoforms exist in two conformations that depend on intact disulfide bonds (our unpublished observations). Finally, immunoprecipitation of cell-surface–biotinylated cells verified that both isoforms were expressed at the cell surface and paired with the β1 integrin subunit (Figure 2B).

Table 1. Flow cytometry expression levels of α7-X1 and α7-X2 in MCF-7 cells

Cell line	Peak area[a] (arbitrary units)
X1-A	64.6 ± 7.2
X1-S3	65.2 ± 1.2
X2-A	65.8 ± 3.7
X2-6	68.9 ± 2.4

[a] Average ±SD of three experiments.

Differential Adhesion of α7-X1 and α7-X2 to Laminin

We compared the ligand binding and specificity of the α7-X1 and α7-X2 isoforms by testing the transfectants in standard adhesion assays using different concentrations of laminin 1 and other ligands. We have shown previously that parental MCF-7 cells express endogenous, potential laminin 1 receptors that are functionally inactive (Yao *et al.*, 1996b). On laminin 1, α7-X2 transfectants attached with high efficiency; adhesion was dependent on the ligand coating concentration (Figure 3A). In previous studies we have shown by using α7-blocking mAbs, that this binding to laminin 1 is specific for α7-X2-expressing MCF-7 cells (Yao *et al.*, 1996b). In contrast, the α7-X1 cell lines, like the parental MCF-7 cells, adhered poorly, even at the highest ligand concentrations. When laminin 1 was replaced with purified laminin 5, both X1 and X2 isoforms failed to bind (our unpublished observations).

The lack of adhesion of the α7-X1 cell lines to laminin 1 suggested that the transfectants might have a general defect in their ability to adhere. To evaluate this possibility, we tested the capacity of the X1 transfectants to bind collagen I. Both X1 and X2 cell lines and parental MCF-7 cells showed similar binding to collagen I, and as expected, antibodies to the α2 collagen receptor completely blocked this adhesion (Figure 3B). Binding to collagen IV showed similar results (our unpublished results).

We also evaluated whether α7-X1 or α7-X2 could bind fibronectin, because it was reported that the α7 subunit binds to this ligand (Gu *et al.*, 1994). However, both X1 and X2 transfectants bound equally to fibronectin (Figure 4). Blocking antibodies to α5 and αv dramatically decreased the ability of the X1 and X2 clones to bind fibronectin, and the combination of α5 and αv blocking antibodies totally inhibited binding to fibronectin for all three cell lines. The parental MCF-7 cells adhered to fibronectin with somewhat higher efficiency than the α7 transfectants, which presumably reflects an α7-induced decrease in the fibronectin receptor expression. We have shown a similar down-regulation of α3β1 receptor expression in MCF-7 cells transfected with α7 (Yao *et al.*, 1996b). Thus, these results show that neither the X1 nor the X2 isoform of the α7 subunit can function as a fibronectin receptor, but cells transfected with either isoform can bind collagen I and IV, indicating that the X1-transfected cells do not have a defect in adherence even though X1 does not bind laminin 1.

α7-X1 Integrin Competency for Adhesion and Spreading

The failure of the X1 isoform to bind laminin suggested that this α7 isoform may be functionally defective. To test for this possibility, we artificially cross-

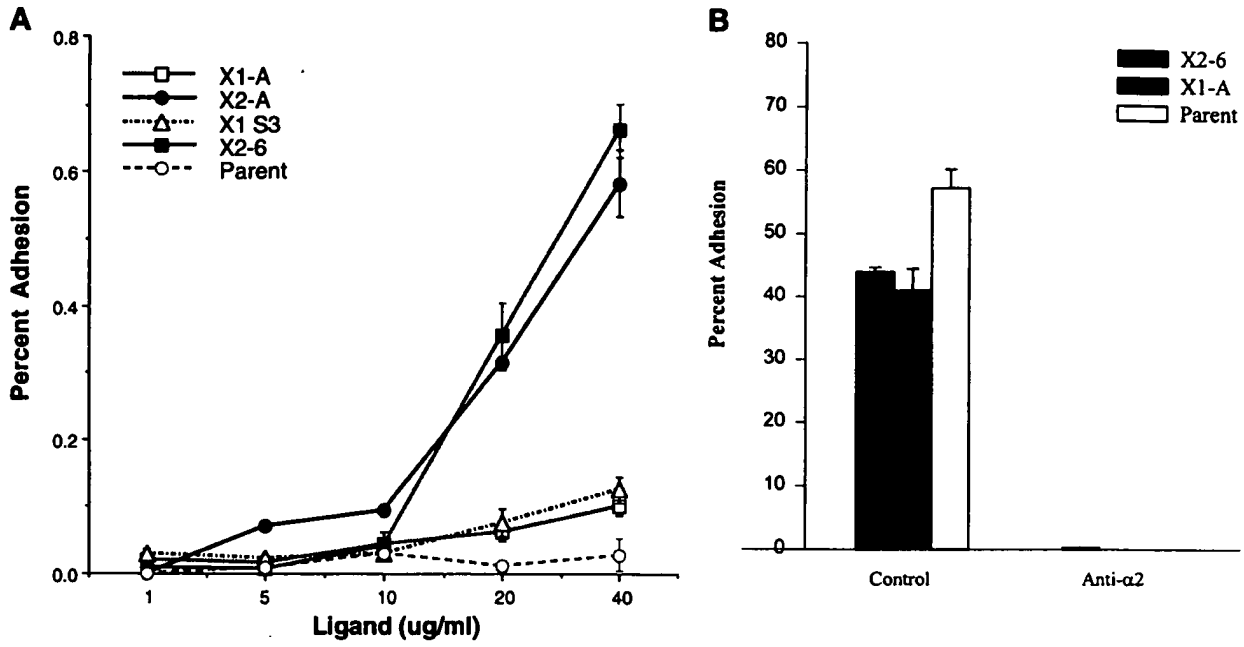


Figure 3. α7-X1 cell lines fail to bind laminin 1. (A) Dose–response curves of cell adhesion to laminin 1. Parental MCF-7, α7-X1, and α7-X2 cells (2×10^4 cells/well) were tested for adhesion to increasing concentrations of laminin 1 as described in MATERIALS AND METHODS. α7-X2 clones showed a dose-dependent adhesion to increasing concentrations of laminin 1. α7-X1 and parental cells bound poorly to laminin at all concentrations. (B) Adhesion of parental MCF-7, α7-X1, and α7-X2 cells to collagen I. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to collagen-I–coated plates (5 μg/ml). Anti-α2 mAb (VM1) was preincubated with cells at 10 μg/ml. Cells bound to collagen type I (at 100 μg/ml) were used to indicate 100% adhesion. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as percentages of the total cells added to each well. (A and B) Values are the mean of triplicate wells; bars indicate the SD.

linked the α7-X1 receptor by using an anti-α7 mAb capture assay. In this assay, mAbs directed against the extracellular domain of the α7 subunit were immobilized on culture dishes (see MATERIALS AND METHODS). Transfectants expressing either α7 isoform were allowed to bind the immobilized mAbs. Both α7-X1 and α7-X2 cell lines attached with equal efficiency to the bound anti-α7 mAb (Figure 5A); when anti-β1 mAb was used, both transfectants attached somewhat more efficiently (our unpublished results). Importantly, both X1 and X2 transfectants were induced to spread within 60 min on the immobilized mAbs (Figure 5, B and C). These results show that both X2 and X1, when cross-linked by anti-α7 mAbs, induce an adhering and spreading response typical of functional integrins that requires assembly of the cytoskeleton. In addition, this suggests that although the α7-X1 integrin is unable to engage laminin, it can still function normally in postligand-binding events.

Solubilized α7-X1 Integrin Fails to Bind Ligand

To determine whether laminin binding by the two isoforms is regulated by their cellular environment, transfectants were processed for ligand affinity chromatography. Receptors were solubilized in detergent and applied to E8-laminin-Sepharose. The presence of α7 in the unbound flow-through fractions and EDTA-eluted fractions was assessed by immunoprecipitation with an α7 mAb. Nearly all of the α7-X2 bound to the E8-Sepharose column and was eluted with EDTA (Figure 6). In contrast, all of the α7-X1 was found in the flow-through and was not detected in the EDTA-eluted fractions (Figure 6). Such a result is consistent with the X1 isoform being functionally unable to engage its ligand even when freed from potential cellular regulatory elements.

The α7-X1 Isoform Can Exist in a Ligand-binding Incompetent State

The antibody-capture adhesion assay indicated that the α7-X1 isoform can function as a bridge between the cytoskeleton and the extracellular matrix. However, it fails to explain why this isoform is deficient in binding its ligand. It is well known that many integrins can alternate between activated and inactive states (reviewed in Hemler *et al.*, 1994; Humphries, 1996; Mould, 1996). This reversible transition to the

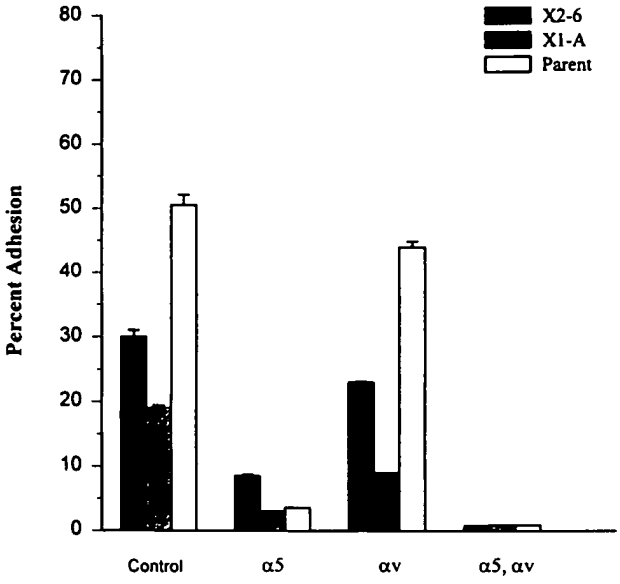


Figure 4. Adhesion of parental MCF-7, α7-X1, and α7-X2 cells to fibronectin. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to fibronectin coated plates (10 μg/ml) as described under MATERIALS AND METHODS. Anti-α5 mAb (P1D6) and anti-αv mAb (LM230) were preincubated with cells at 10 μg/ml. Cell adhesion was determined as detailed in Figure 3.

active state is associated with enhanced ligand-binding affinity and is believed to depend on conformational changes that can be influenced by several factors including stimulatory or activating anti-integrin antibodies and divalent cations (Humphries, 1996). To test for the possibility that the inability of α7-X1 to bind ligand is a result of the receptor's activation state, we performed adhesion assays to laminin 1 in the presence or absence of β1 activating antibodies. Addition of the activating anti-β1 mAb TS2/16 substantially increased the adhesion to laminin 1 for α7-X1, α7-X2, and parental MCF-7 cell lines (Figure 7). The most dramatic increase occurred in the α7-X1 clone, which went from minimal adhesion in the absence of activating antibody to nearly the same level as the α7-X2 cell line in the presence of mAb TS2/16.

To identify the integrin complexes responsible for α7-X1's increased adhesion, we repeated the adhesion assays with TS2/16 and a combination of integrin-blocking antibodies. A mixture of blocking antibodies to integrin subunits α2, α3, and α6 completely inhibited TS2/16-activated MCF-7 parental cells from binding to laminin 1 (Figure 7). In contrast, these same blocking antibodies only slightly reduced the TS2/16-activated α7-X1 and α7-X2 cell adhesion to laminin, suggesting that this adhesion was mediated by the α7 subunit. When blocking antibodies to α7 were added to the anti-α2, -α3, and -α6 mAb mixture, adhesion to laminin 1 was completely inhibited for the α7-X1 and α7-X2 cell lines (Figure 7). Similar results were seen when TS2/16 was replaced with the 8A2 activating mAb but not with 9EG7 or 15/7 mAbs (our unpublished results). Results again were similar when these experiments were repeated with the laminin E8 fragment, which contains the α7 binding site (our unpublished results). Interestingly, TS2/16-activated α7-X2

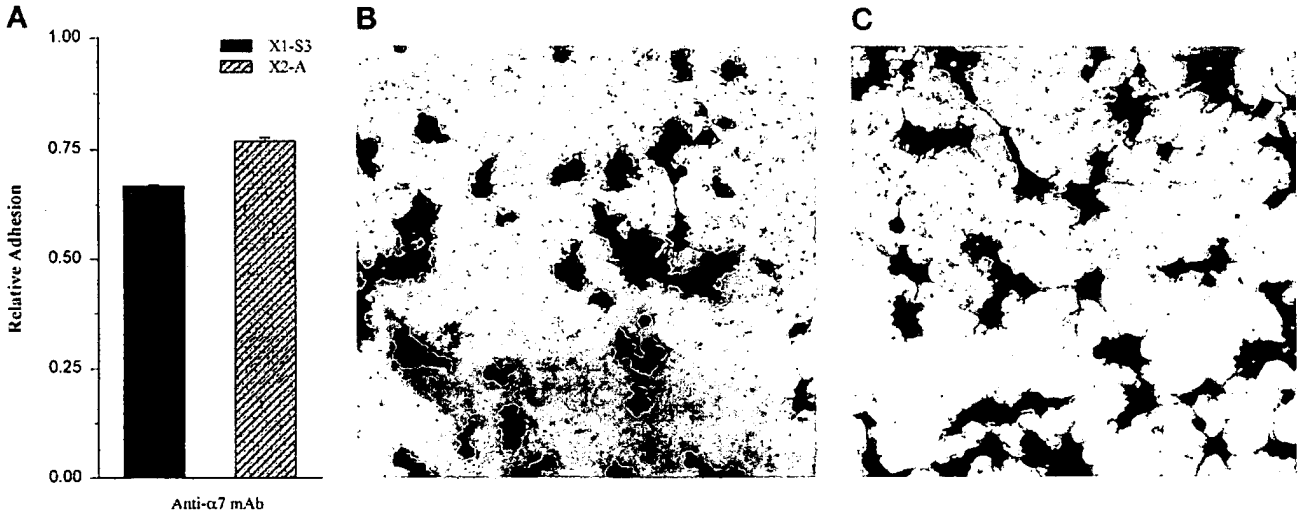


Figure 5. α7-X1 expressing cells adhere and spread on anti-α7. (A) Microtiter plates (96-well) were coated with anti-α7 mAb at 15 μg/ml in PBS. Plates were washed with PBS and incubated with DMEM containing 0.1% BSA to block nonspecific adhesion. Single cell suspensions were prepared in DMEM with 0.1% BSA; 2×10^4 cells were added to each well in triplicate in 96-well plates and incubated for 60 min at 37°C. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as relative adhesion based on optical density (OD) units. Values are the mean of triplicate wells; bars indicate the SD. Both α7-X1 (B) and α7-X2 (C) cells have attached and spread on immobilized anti-α7 mAbs. After 1 h cells were fixed with 1% formaldehyde and stained with 1% crystal violet.

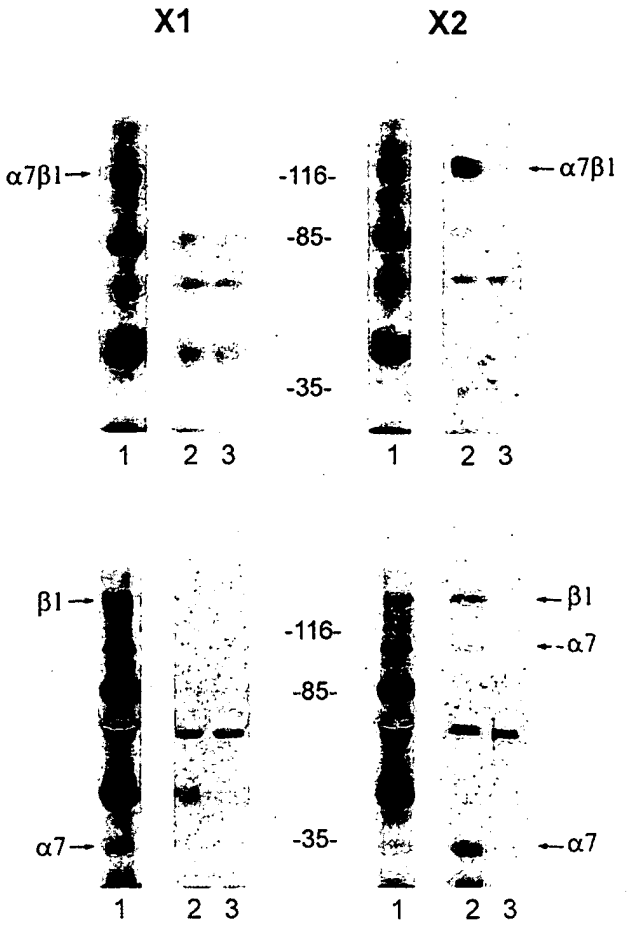


Figure 6. Detergent-solubilized α7-X2 is able to bind immobilized E8 fragment of laminin 1. Cell-surface biotin-labeled α7-X1– and α7-X2–transfected MCF-7 cells were lysed in OPG and processed for ligand affinity chromatography on laminin E8-Sepharose columns. The unbound flow-through material was collected, and after extensive washing of the column, bound integrin was eluted with EDTA (see MATERIALS AND METHODS). The material present in the crude flow-through fraction (lane 1) and the first two fractions eluted with EDTA (lanes 2 and 3) were processed for immunoprecipitation with anti-α7 antibody (1211) and analyzed by SDS-PAGE under nonreduced (top) or reduced conditions (bottom). The positions of the α7 and β1 subunits are indicated. Note that the α7 subunit separates into its heavy and light chains after reduction (Yao *et al.*, 1996b).

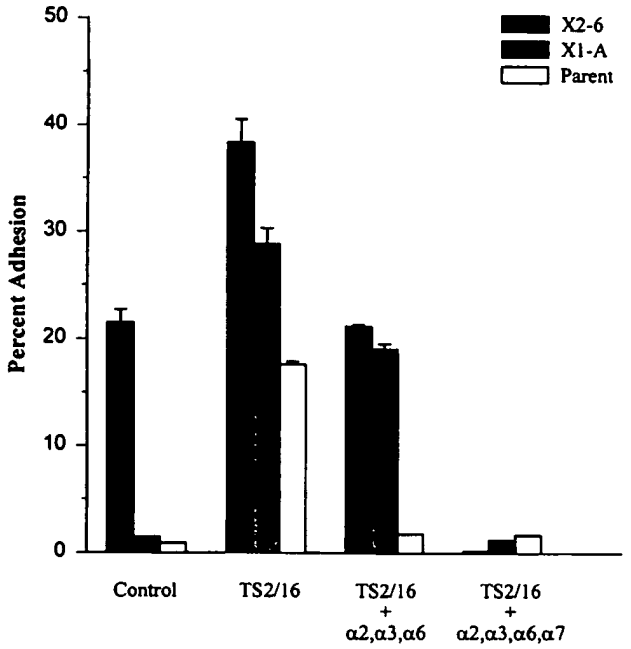


Figure 7. Stimulation of α7-X1 laminin binding activity by mAb TS2/16. (A) Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated wells (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with TS2/16 (1.5 μg/ml) and combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), all at 10 μg/ml. Cell adhesion was determined as detailed in Figure 3.

cells in the presence of anti-α2, -α3, and -α6 adhered at nearly the same level as the untreated α7-X2 transfectants. Thus, these results clearly demonstrate that the failure of the α7-X1 isoform to bind laminin 1 is linked to the activation state of the subunit, which in turn is regulated by the swapping of X2 and X1 inserts.

Mn^{2+} Fails to Activate α7-X1 Adhesion

Ligand binding by integrins is dependent on divalent cations (Sanchez-Mateos *et al.*, 1996). Work by a number of investigators has shown that Mn^{2+}, and to a lesser extent Mg^{2+}, can enhance ligand binding by switching the integrin to an activated state via a mechanism believed to be similar to that produced by activating β1 antibodies (Humphries, 1996; Mould, 1996). When Mn^{2+} replaced Ca^{2+}/Mg^{2+} as the divalent cation in adhesion assays, the parental, X1, and X2 cell lines all displayed an increase in adhesion to laminin (Figure 8). However, the Mn^{2+}-induced increase in adhesion seen for X1 was inhibited by a mixture of blocking antibodies to integrins α2, α3, and α6, indicating that α7-X1 is resistant to Mn^{2+}-induced activation. As with the TS2/16-treated cells, Mn^{2+} had only a slight stimulating effect on laminin 1 binding for α7-X2, suggesting that the X2 isoform is already fully activated. These results are consistent with those of the E8-Sepharose chromatography assays, which used detergent-solubilized α7 receptor in the presence of Mn^{2+}.

Regulation of α7-X1 Ligand Competency Is Cell Type Specific

It is now appreciated that individual integrins can display altered levels of activation depending on the

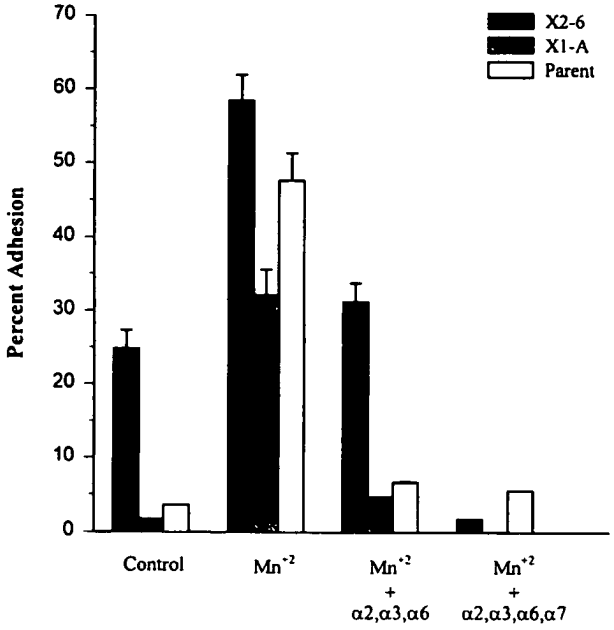


Figure 8. α7-X1 is not cation dependent for laminin 1 adhesion. Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated plates (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with Mn^{2+} (0.1 mM) and combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

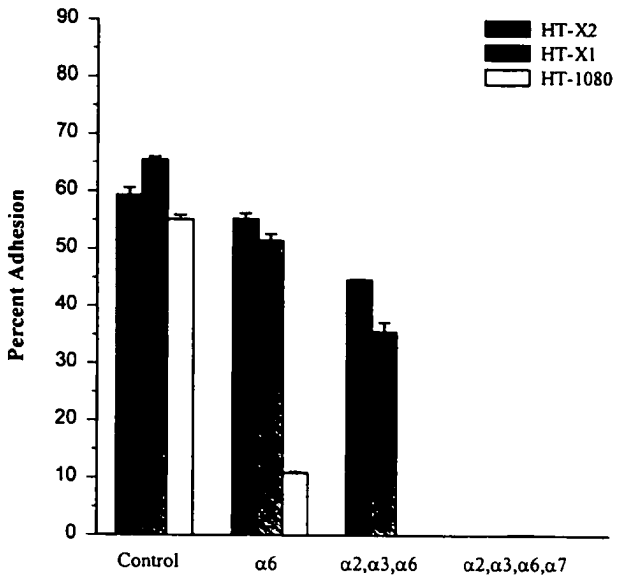


Figure 9. Adhesion of HT1080 parental and HT1080 α7-X1 and α7-X2 cell lines to laminin 1. Parental HT1080 cells were transfected with α7-X1 and α7-X2 as described in MATERIALS AND METHODS. HT1080, HT1080X1, and HT1080X2 cell lines (2×10^4 cells) were added to laminin 1-coated plates (15 μg/ml) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with combinations of blocking antibodies α2 (VM1), α3 (P1B5), α6 (GoH3), and α7 (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

cell type (Gehlsen *et al.*, 1989; Chan and Hemler, 1993). When we expressed the α7-X1 or α7-X2 variant integrins in MCF-7 cells, the two receptors showed differential binding activity for laminin 1. MCF-7 cells appear to be in the class of host cells that confer a poor level of integrin activation, as indicated by the fact that endogenously expressed α2 and α6 as well as α7-X1 integrins in MCF-7 cells are not capable of binding laminin 1, yet can be fully activated by mAb TS2/16 (Figure 7; Yao *et al.*, 1996b). For an activation-permissive cell, we chose the HT1080 human fibrosarcoma cells. This cell line expresses a fully functional α6β1 integrin, and adhesion to laminin 1 can be blocked by mAb to α6 (GoH3; Lin *et al.*, 1993; Matter and Laurie, 1994). For analysis of the cell-type-dependent activation of α7, HT1080 cells were transfected with the α7-X1 or α7-X2 isoform, and high-expressing subpopulations of each transfectant were enriched for α7 by flow cytometry after an initial selection with G418. Both the X1- and X2-transfected cell lines as well as the parent HT1080 cells showed strong adhesion to laminin (Figure 9). However, after treatment with antibodies to α6, the binding of the parental cells was greatly reduced and was completely inhibited in the presence of mAbs to α2, α3, and α6. Although the X1 and X2 transfectants also showed partial blocking of adhesion to laminin in the presence of this mixture of mAbs, substantial binding was still evident, but this binding was abolished when α7 mAb was included. This indicates that in contrast to α7-X1 expression in MCF-7 cells, the integrin's expression in HT1080 cells leads to activation levels that are nearly equivalent to those of the α7-X2 variant.

DISCUSSION

In this study we investigated possible functional differences between X1 and X2, two variants of the α7 integrin subunit that are generated by alternative mRNA splicing in the extracellular domain of the receptor. Our findings show that the alternatively spliced X1 sequence modifies the activation state of the integrin. Whereas the α7-X2 isoform is constitutively active in both cell types, the X1 variant is inactive in MCF-7 cells and fully active in HT1080 cells. This is the first example of cell-type regulation of integrin function that is controlled by alternative splicing in the extracellular subunit domain.

Several α integrin subunits can be alternatively spliced in the extracellular domain between the III and IV N-terminal repeat domains, a region critically important for ligand binding (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). Alternative

splicing in *Drosophila* integrin αPS2 has been shown to alter the binding efficiency of the receptor to the RGD-dependent ligands fibronectin and vitronectin (Brown *et al.*, 1989; Zavortink *et al.*, 1993; Roote and Zusman, 1996). The α6 subunit is also alternatively spliced; however, unlike α7, alternative splicing in α6 results in either exon X1 or a combination of both exons X1 and X2 being spliced in. In contrast to α7, the X1 and X1X2 isoforms of α6 showed no difference in ligand affinity, specificity, or integrin activation. Furthermore, α6-X1X2 is found as a rare mRNA and is probably not physiologically relevant, whereas expression of the α6-X2 isoform has not been detected (Delwel *et al.*, 1995). Loss of α6-X2 expression probably reflects an evolutionary change in the primordial α7-like gene that is believed to have given rise to α6. It has been suggested that the α3 subunit is also alternatively spliced extracellularly, but the X1 isoform for this subunit has yet to be described (Ziober *et al.*, 1993). In contrast to α6, both α7 alternatively spliced isoforms X1 and X2 are expressed (Ziober *et al.*, 1993; Wang *et al.*, 1995). More importantly, this alternative splicing, as shown herein, regulates the α7 receptor's ability to bind ligand.

Since α7-X1 lacks detectable laminin 1 binding activity, we postulated that the α7-X1 variant is expressed in an inactive but presumably reversible conformation, an event that can occur in other integrins. Furthermore, it is now appreciated that individual integrins can display altered levels of activation that are dependent on cell-type specificity (Hemler *et al.*, 1994). When we expressed the α7-X1 or α7-X2 variant integrins in MCF-7 cells, they showed differential binding activity for laminin 1 (Figure 3A). Endogenously expressed α2 and α6 integrins are not capable of binding laminin 1, indicating that MCF-7 cells appear to be a class of host cells that confers a poor level of integrin activation (Yao *et al.*, 1996b). These two integrins also behave similarly when transfected into the nonpermissive K562 cells (Chan and Hemler, 1993; Delwel *et al.*, 1993, 1995; Kawaguchi and Hemler, 1993). In contrast, the α3 subunit (the X2 form), when transfected into K562 cells, is constitutively active for binding laminin 5, as is endogenous α3 in MCF-7 cells (Weitzman *et al.*, 1993; Delwel *et al.*, 1994). This indicates that the α3 integrin is constitutively active even in cells such as K562 and MCF-7. Thus we consider the α3 integrin to be similar to α7-X2 with regard to its activation state and its sequence homology at the III/IV variable region. In contrast, we suggest that the normally expressed form of α6, the α6-X1 homologue, is cell type specific for activation because of its high homology to α7-X1. HT1080 human fibrosarcoma cells appear to be an X1-activation–permissive cell line because they express constitutively activated α6β1. Thus, α7-X1 and α7-X2, when transfected into HT1080 cells, were both active, indicating that activation of the X1 isoform, either for α6 or α7, depends on cell-type specificity (Figure 9).

The functional state of an integrin complex is dependent on the conformation of its extracellular domain. A number of factors have been shown to modulate the activity of this extracellular conformation (Humphries, 1996; Mould, 1996). For example, integrins are sensitive to divalent cation occupancy, and it is well established that cations can regulate integrin activity (Humphries, 1996). In particular, low concentrations of Mn^{2+} have been shown to induce a high ligand affinity state in integrins (Schwartz *et al.*, 1995). In our studies with the MCF-7 transfectants, Mn^{2+} was able to activate only the α2 and α3 integrins and not the α7-X1 isoform. However, Mn^{2+} did not appear to elevate X2 activity, suggesting that Ca^{2+}/Mg^{2+} are sufficient for a fully functional α7. This suggests that insertion of the X1 exon appears to alter the conformation of the subunit (which probably accounts for the ~3300-kDa molecular mass change in X1 as compared with X2, Figure 2). Such a conformational change may possibly affect cation binding. Similar studies with α2 showed that cations could not convert the VLA-2 form-O or form-C into a more active form but activating antibodies could (Chan and Hemler, 1993).

In general, activating antibodies appear to increase the ligand binding competency of integrin molecules. We have shown in this study that the X1 isoform is competent to bind laminin 1 only when activated by mAbs TS2/16 or 8A2 (Figure 7; our unpublished results). TS2/16 and 8A2 bind to the same epitope and activate the β1 integrin subunit by altering its conformation (Faull *et al.*, 1993; Humphries, 1996). In contrast, 9EG7 was not able to induce an active conformation in the X1 isoform (our unpublished results). This antibody recognizes an epitope, distinct from that of TS2/16 and 8A2, that is present when β1 is activated by Mn^{2+} (Bazzoni *et al.*, 1995; Humphries, 1996). 9EG7 is believed not to define an activation epitope but a ligand-induced binding site that can be induced by cations like Mn^{2+} (Bazzoni *et al.*, 1995). Perhaps the failure of 9EG7 to activate the α7-X1 isoform is also due to this isoform's inability to be activated by Mn^{2+}. Antibody 15/7 also failed to activate α7-X1; it too is considered a Mn^{2+}-inducible anti-β1 antibody (Picker *et al.*, 1993; Puzon-McLaughlin *et al.*, 1996). These results and the failure of X1 to bind laminin 1 in the presence of Mn^{2+} support the notion that the X1 segment is able to alter the conformation of the subunit and inhibit cation binding and ligand adherence.

Comparison of the X1 and X2 alternative splice domains indicates a completely divergent amino acid sequence (Ziober *et al.*, 1993). Recent observations have indicated that critical residues for ligand-binding activity are clustered in a predicted β-turn of the C-terminal portion of the third repeat domain of inte-

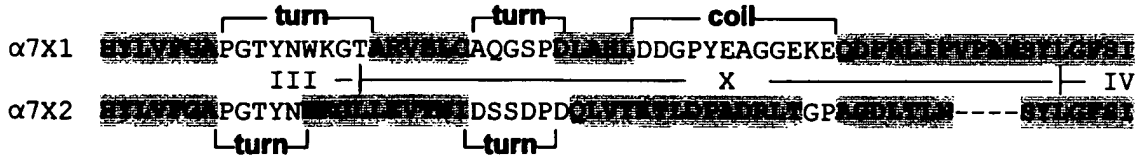


Figure 10. Amino acid sequence and secondary structure of the α7-X1 and X2 alternatively spliced domains. The amino acid sequences for X1 and X2 domains were analyzed by using the MacVector program. β-sheet conformations are boxed and β-turn or random coil regions are indicated. Both X1 and X2 have extensive β-sheet structures; however, X1 has a long random coil motif absent in the X2 that could act as a hinge.

grins α4, α5, and αIIb (Irie *et al.*, 1995; Kamata *et al.*, 1995). This site (GAPGTYNWKG) is conserved in all α-chains, including α7, and is immediately proximal to the α7 X1/X2 domains. This points to the possibility that the region defined by X1/X2, which directly borders the third repeat domain, may be important in regulating integrin activation. The β-turn at this site has been predicted by using several secondary structure prediction methods and from the alignment of the seven repeat domains from 16 different integrin sequences (Tuckwell *et al.*, 1994). Comparison of the α7-X1 and α7-X2 sequences shows a significant structural difference in the C-terminal half of the variable region (Figure 10). Although the X2 region seems to consist of multiple β-sheet structures that apparently align with β-sheet segments in the adjacent IV repeat domain, the X1 region contains a random coil structure of at least 10 residues that begin in the C-terminal half of this variable region. This coil motif in X1 may provide a "hinge-like" structure that may control conformational changes in this putative ligand-binding region of the α7 subunit (Figure 10). Such regulation may ultimately affect ligand-binding competency by turning on or off the subunit's ability to bind to divalent cations. Finally, as shown in this report, this apparent hinge-like structure can be modulated by β1-activating antibodies such as TS2/16.

How is the activation level of the α7 variants regulated? The observation that β1 activation mAbs can convert the α7-X1 isoform to the "on" state suggests that ligand-binding activity can be controlled by alterations in the partner subunit conformation. Affinity modulation by inside-out signaling via transmembrane conformational transitions has been observed previously (reviewed in Schwartz *et al.*, 1995). Since the α7 subunit expression is limited to a few highly differentiated tissues and the expression of the X1/X2 isoforms is developmentally regulated, it is possible that the functionality of this integrin needs to be controlled in a tissue-specific manner. Previous work has shown that α7β1 is expressed during myoblast differentiation and at the myotendinous and neuromuscular junctions in adult skeletal muscle (Song *et al.*, 1992; Ziober *et al.*, 1993; Martin *et al.*, 1996). Both isoforms are expressed in myoblasts, but only X2 is detectable in mature muscle, implying that the constitutively active form of the receptor is present at permanent myotendinous junctions and neuromuscular sites (Song *et al.*, 1992; Ziober *et al.*, 1993; Crawley *et al.*, 1996; Martin *et al.*, 1996). We propose that the regulatable X1 isoform is important during dynamic adhesion situations related to muscle development (motility, fusion, remodeling, repair, and matrix assembly) and that the X2 variant performs more stable adhesion functions (costamers and myotendinous junctions). The function of the two isoforms in skeletal myoblast/myotube adhesion is currently under investigation. Finally, the ras/raf/MAPK signaling pathway has lately been implicated in regulating the integrin affinity state (Hughes *et al.*, 1997). Whether this pathway plays an "inside-out" role in regulating the X1 isoform or even in cell-specific activation of this isoform remains to be determined.

ACKNOWLEDGMENTS

We thank Evangeline Leash for editorial assistance. This work was supported by National Institutes of Health grant DE-10306 to R.H.K.

REFERENCES

Arroyo, A.G., Sanchez-Madrid, P., Campamero, M.R., Martin-Padura, I., Dejana, E., and Sanchez-Madrid, F. (1992). Regulation of the VLA integrin-ligand interactions through the β1 subunit. J. Cell Biol. *117*, 659–670.

Bazzoni, G., Shih, D.T., Buck, C.A., and Hemler, M.E. (1995). Monoclonal antibody 9EG7 defines a novel β1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. J. Biol. Chem. *270*, 25570–25577.

Belkin, A.M., Zhidkova, N.I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V.E., and Burridge, K. (1996). Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. J. Cell Biol. *132*, 211–226.

Brown, N.H., King, D.L., Wilcox, M., and Kafatos, F.C. (1989). Developmentally regulated alternative splicing of *Drosophila* integrin PS2 alpha transcripts. Cell *59*, 185–195.

Chan, B.M.C., and Hemler, M.E. (1993). Multiple functional forms of the integrin VLA-2 can be derived from a single α2 cDNA clone: interconversion of forms induced by an anti-β1 antibody. J. Cell Biol. *120*, 537–543.

Collo, G., Star, L., and Quaranta, V. (1993). A new isoform of the laminin receptor integrin α7β1 is developmentally regulated in skeletal muscle. J. Biol. Chem. *268*, 19019–19024.

Cooper, H.M., Tamura, R.N., and Quaranta, V. (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the alpha 6 beta 1 integrin. J. Cell Biol. *115*, 843–850.

Crawley, S.C., Kaufman, S.J., and Cooper, D.N.W. (1996). Comparison of rat myoblast receptors for laminin-1 and laminin-2–4. Mol. Biol. Cell *7*(suppl), 67a. (Abstract).

Delwel, G.O., *et al.* (1994). Distinct and overlapping ligand specificities of the alpha 3A beta 1 and alpha 6A beta 1 integrins: recognition of laminin isoforms. J. Biol. Chem. *268*, 25865–25875.

Delwel, G.O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R., and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin alpha 6 subunit in transfected K562 cells. Activation-dependent adhesion and interaction with isoforms of laminin. J. Biol. Chem. *268*, 25865–25875.

Delwel, G.O., Kuikman, I., and Sonnenberg, A. (1995). An alternatively spliced exon in the extracellular domain of the human alpha 6 integrin subunit–functional analysis of the alpha 6 integrin variants. Cell Adh. Comm. *3*, 143–161.

Diamond, M.S., and Springer, T.A. (1994). The dynamic regulation of integrin adhesiveness. Curr. Biol. *4*, 506–517.

Djaffar, I., Chen, Y.P., Creminon, C., Maclouf, J., Cieutat, A.M., Gayet, O., and Rosa, J.P. (1994). A new alternative transcript encodes a 60 kDa truncated form of integrin beta 3. Biochem. J. *300*, 69–74.

Faull, R.J, Kovach, N.L., Harlan, J.M., and Ginsberg, M.H. (1993). Affinity modulation of integrin alpha 5 beta 1: regulation of the functional response by soluble fibronectin. J. Cell Biol. *121*, 155–162.

Fornaro, M., Zheng, D.Q., and Languino, L.R. (1995). The novel structural motif Gln795-Gln802 in the integrin beta 1C cytoplasmic domain regulates cell proliferation. J. Biol. Chem. *270*, 24666–24669.

Gehlsen, K.R., Dickerson, K., Argraves, W.S., Engvall, E., and Ruoslahti, E. (1989). Subunit structure of laminin-binding integrin and localization of its binding site on laminin. J. Biol. Chem. *264*, 19034–19038.

Grinblat, Y., Zusman, S., Yee, G., Hynes, R.O., and Kafatos, F.C. (1994). Functions of the cytoplasmic domain of the beta PS integrin subunit during *Drosophila* development. Development *120*, 91–102.

Gu, M., Wang, W., Song, W.K., Cooper, D.N.W., and Kaufman, S.J. (1994). Selective modulation of the interaction of α7β1 integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. J. Cell. Sci. *107*, 175–181.

Hemler, M.E., Weitzman, J.B., Pasqualini, R., Kawaguchi, S., Kassner, P.D., and Berdichevsky, F.B. (1994). Structure, biochemical properties, and biological functions of integrin cytoplasmic domains. In: Integrins: The Biological Problems, ed. Y. Takada, Boca Raton, FL: CRC Press, 1–35.

Hogervorst, F., Kuikman, I., van Kessel, A.G., and Sonnenberg, A. (1991). Molecular cloning of the human alpha 6 integrin subunit. Alternative splicing of alpha 6 mRNA and chromosomal localization of the alpha 6 and beta 4 genes. Eur. J. Biochem. *199*, 425–433.

Hughes, P.E., Renshaw, M.W., Pfaff, M., Forsyth, J., Keivens, V.M., Schwartz, M.A., and Ginsberg, M.H. (1997). Suppression of integrin activation: a novel function of a ras/raf-initiated MAP kinase pathway. Cell *88*, 521–530.

Humphries, M.J. (1996). Integrin activation: the link between ligand binding and signal transduction. Curr. Opin. Cell Biol. *8*, 632–640.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Irie, A., Kamata, T., Puzon-McLaughlin, W., and Takada, Y. (1995). Critical amino acid residues for ligand binding are clustered in a predicted β-turn of the third N-terminal repeat in the integrin α4 and α5 subunits. EMBO J. *14*, 5550–5556.

Kamata, T., Puzon, W., and Takada, Y. (1995). Identification of putative ligand-binding sites of the integrin alpha 4 beta 1 (VLA-4, cd49d/CD29). Biochem. J. *305*, 945–951.

Kawaguchi, S., and Hemler, M.E. (1993). Role of the alpha subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. J. Biol. Chem. *268*, 16279–16285.

Kramer, R.H., Vu, M.P., Cheng, Y.F., Ramos, D.M., Timpl, R., and Waleh, N. (1991). The laminin-binding integrin α7β1: functional characterization and expression in normal and malignant melanocytes. Cell. Regul. *2*, 805–817.

Kramer, R.H. (1994). Isolation and characterization of integrin laminin receptors. Methods Enzymol. *245*, 129–147.

Languino, L.R., and Ruoslahti, E. (1992). An alternative form of the integrin beta 1 subunit with a variant cytoplasmic domain. J. Biol. Chem. *267*, 7116–7120.

Lin, C.S., Zhang, K., and Kramer, R. (1993). Alpha 6 integrin is up-regulated in step increments accompanying neoplastic transformation and tumorigenic conversion of human fibroblasts. Cancer Res. *53*, 2950–2953.

Martin, P.T., Kaufman, S.J., Kramer, R.H., and Sanes, J.R. (1996). Synaptic integrins in developing, adult, and mutant muscle: selective association of alpha1, alpha7A, and alpha7B integrins with the neuromuscular junction. Dev. Biol. *174*, 125–139.

Matter, M.L., and Laurie, G.W. (1994). A novel laminin E8 cell adhesion site required for lung alveolar formation in vitro. J. Cell Biol. *124*, 1083–1090.

Meredith, J., Jr., Takada, Y., Fornaro, M., Languino, L.R., and Schwartz, M.A. (1995). Inhibition of cell cycle progression by the alternatively spliced integrin beta 1C. Science *269*, 1570–1572.

Mould, P.A. (1996). Getting integrin into shape: recent insights into how integrin activity is regulated by conformational changes. J. Cell Sci. *109*, 2613–2618.

Picker, L.J., Treer, J.R., Nguyen, M., Terstappen, L.W., Hogg, N., and Yednock, T. (1993). Coordinate expression of beta 1 and beta 2 integrin "activation" epitopes during T cell responses in secondary lymphoid tissue. Eur. J. Immunol. *23*, 2751–2757.

Puzon-McLaughlin, W., Yednock, T.A., and Takada, Y. (1996). Regulation of conformation and ligand binding function of integrin alpha5beta1 by the beta1 cytoplasmic domain. J. Biol. Chem. *271*, 16580–16585.

Roote, C.E., and Zusman, S. (1996). Alternatively spliced forms of the Drosophila αps1 subunit if integrin are sufficient for viability and can replace the function of the αps1 subunit if integrin in the retina. Development *122*, 1985–1994.

Sanchez-Mateos, P., Cabanas, C., and Sanchez-Madrid, F. (1996). Regulation of integrin function. Cancer Biol. *7*, 99–109.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol. *11*, 549–599.

Shimizu, Y., Van Seventer, G.A., Horgan, K.J., and Shaw, S. (1990). Regulated expression and function of three VLA (beta 1) integrin receptors on T cells. Nature *345*, 250–253.

Song, W.K., Wang, W., Sato, H., Bielser, D.A., and Kaufman S.J. (1993). Expression of alpha 7 integrin cytoplasmic domains during skeletal muscle development: alternate forms, conformational change, and homologies with serine/threonine kinases and tyrosine phosphatases. J. Cell Sci. *106*, 1139–1152.

Song, W.K., Wang, W., Foster, R.F., Bielser, D.A., and Kaufman, S.J. (1992). H36-α7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. J. Cell. Biol. *117*, 643–657.

Suzuki, S., and Naitoh, Y. (1990). Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. EMBO J. *9*, 757–763.

Takada, Y., and Puzon, W. (1993). Identification of a regulatory region of integrin β1 subunit using activating and inhibiting antibodies. J. Biol. Chem. *268*, 17597–17601.

Tamura, R.N., Cooper, H.M., Collo, G., and Quaranta, V. (1991). Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. Proc. Natl. Acad. Sci. USA *88*, 10183–10187.

Tennenbaum, T., Belanger, A.J., Glick, A.B., Tamura, R., Quaranta, V., Yuspa, S.H. (1995). A splice variant of alpha 6 integrin is associated with malignant conversion in mouse skin tumorigenesis. Proc. Natl. Acad. Sci. USA *92*, 7041–7045.

Tuckwell, D.S., Humphries, M.J., and Brass, A. (1994). A secondary structure model of the integrin alpha subunit N-terminal domain based on analysis of multiple alignments. Cell Adh. Comm. *2*, 385–402.

van der Flier, A., Kuikman, I., Baudoin, C., van der Neut, R., and Sonnenberg, A. (1995) A novel beta 1 integrin isoform produced by alternative splicing: unique expression in cardiac and skeletal muscle. FEBS Lett. *369*, 340–344.

van Kuppevelt, T.H., Languino, L.R., Gailit, J.O., Suzuki, S., and Ruoslahti, E. (1989) An alternative cytoplasmic domain of the integrin beta 3 subunit. Proc. Natl. Acad. Sci. USA *86*, 5415–5418.

Wang, W., Wu, W., Desai, T., Ward, D.C., and Kaufman, S.J. (1995). Localization of the alpha 7 integrin gene (ITGA7) on human chromosome 12q13: clustering of integrin and Hox genes implies parallel evolution of these gene families. Genomics *26*, 568–570.

Weitzman, J.B., Pasqualini, R., Takada, Y., and Hemler, M. (1993). The function of distinct regulation of the integrin VLA-3 in cell adhesion, spreading, and homotypic cell aggregation. J. Biol. Chem. *268*, 8651–8657.

Yao, C.-C., Ziober, B.L., Sutherland, A.E., Mendricks, D.L., and Kramer, R.H. (1996a). Laminins promote the locomotion of skeletal myoblasts via the alpha 7 integrin receptor. J. Cell Sci. *109*, 3139–3150.

Yao, C.-C., Ziober, B.L., Squillace, R.M., and Kramer, R.H. (1996b). Alpha7 integrin mediates cell adhesion and migration on specific laminin isoforms. J. Biol. Chem. *271*, 25598–25603.

Yao, C.-C., Breuss, J., Pytela, R., and Kramer, R.H. (1997). Functional expression of the alpha 7 integrin receptor in differentiated smooth muscle cells. J. Cell Sci. *110*, 1477–1487.

Zavortink, M., Bunch, T.A., and Brower, D.L. (1993). Functional properties of alternatively spliced forms of the *Drosophila* PS2 integrin alpha subunit. Cell Adh. Comm. *1*, 251–264.

Zhidkova, N.I., Belkin, A.M., and Mayne, R. (1995). Novel isoform of beta 1 integrin expressed in skeletal and cardiac muscle. Biochem. Biophys. Res. Commun. *214*, 279–285.

Ziober, B.L., Vu, M.P., Waleh, N., Crawford, J., Lin, C.-S., and Kramer, R.H. (1993). Alternative extracellular and cytoplasmic domains of the integrin α7 subunit are differentially expressed during development. J. Biol. Chem. *268*, 26773–26783.

APPENDIX 3

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Ziober et al., Mol Biol Cell. 1997 Sep;8(9):1723-34 (see Appendix 1)

Erle et al., J Biol Chem. 1991 Jun 15;266(17):11009-16. (8 pages)

Humphries et al., Ciba Found Symp. 1995;189:177-91; discussion 191-9. (24 pages)

Shaw and Mercurio, Mol Biol Cell. 1994 Jun;5(6):679-90. (12 pages)

Djaffar et al., Biochem J. 1994 May 15;300 (Pt 1):69-74. (6 pages)

Collo et al., J Biol Chem. 1993 Sep 5;268(25):19019-24. (6 pages)

Song et al., J Cell Sci. 1993 Dec;106 (Pt 4):1139-52. (14 pages)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 266, No. 17, Issue of June 15, pp. 11009-11016, 1991
Printed in U.S.A.

Complete Amino Acid Sequence of an Integrin β Subunit (β_7) Identified in Leukocytes*

(Received for publication, January 15, 1991)

David J. Erle‡, Curzio Rüegg§, Dean Sheppard, and Robert Pytela

From the Lung Biology Center, the Cardiovascular Research Institute, and the Department of Medicine, University of California, San Francisco, California 94143

The integrins are a large family of heterodimeric cell-surface glycoproteins that play key roles in the adherence of cells to other cells and to extracellular matrix proteins. We have previously reported the identification of a novel integrin β subunit partial cDNA from leukocytes. We have now determined the complete sequence of this subunit, designated as β_7, from overlapping clones obtained from a PEER T leukemia cell library and a peripheral T cell library. The β_7 cDNA contains a single large open reading frame predicted to encode a 798-amino acid protein precursor (signal peptide plus mature protein). The β_7 protein, like the other β subunit proteins, is predicted to contain a large extracellular portion, a transmembrane domain, and a cytoplasmic tail. The deduced β_7 amino acid sequence is 32–46% identical to the six previously sequenced human integrin β subunits. β_7 is most similar to the leukocyte integrin common β subunit (β_2, CD18). Analysis of variant β_7 cDNA clones and reverse transcription-polymerase chain reaction products suggest that alternatively spliced β_7 mRNAs can be generated by the removal of exons that encode most of the cysteine-rich region of the extracellular portion of β_7. By Northern blot analysis, β_7 mRNA was detected in T and B cell lines and in macrophage-like cell lines, but not in any of the nonleukocyte cell lines tested. Peripheral T cells and some lymphoma lines express little β_7 mRNA before stimulation; but after stimulation with phorbol ester, β_7 mRNA levels increased markedly. Integrin β_7 is expected to play a role in adhesive interactions of leukocytes.

Integrins are a family of heterodimeric cell-surface glycoproteins that mediate adhesion of cells to other cells and to extracellular matrix (1). Leukocytes express a variety of integrins that are crucial participants in the inflammatory and immune responses. Patients deficient in the leukocyte integrins LFA-1 ($\alpha_1\beta_2$), Mac-1 ($\alpha_M\beta_2$), and p150,95 ($\alpha_X\beta_2$) are unable to mount a normal inflammatory response because leukocytes fail to extravasate and enter tissues at sites of infection or injury (2). Activation of T cells results in expression of a variety of integrins at the cell surface, and the integrins LFA-1 and VLA-4 ($\alpha_4\beta_1$) each play a role in lymphocyte-lymphocyte and lymphocyte-target cell interactions (3, 4). In addition, the integrin LPAM-1 ($\alpha_4\beta_P$) functions as a lymphocyte homing receptor (5, 6).

All integrins are composed of one α subunit and one β subunit that are translated separately and are noncovalently associated. The cDNA sequences of six mammalian β subunits, β_1–β_6, have been reported previously (7–16). The β_2 subunit (CD18) is expressed only on leukocytes; other β subunits, for example β_1, are expressed on leukocytes and on many other cell types. There are striking overall structural similarities among these six β subunits. In some short regions, there is almost complete conservation of amino acid sequence between different β subunits. We previously described a method for the amplification of known and novel integrin cDNAs using the polymerase chain reaction (PCR)[1] and oligonucleotide primers that recognize highly conserved integrin sequences (16, 17). As previously reported, we used this homology PCR technique to amplify cDNA made from two human lymphocyte lines (MoLT-4 and Raji) and identified a 292-nucleotide novel cDNA (17). The novel cDNA is predicted to encode a 97-amino acid fragment of an integrin β subunit, designated β_7, that is 40–61% identical to the corresponding fragments of the six previously sequenced β subunits. We amplified similar partial cDNAs that presumably encode the mouse and rabbit homologs of β_7 from mouse lymphocyte and macrophage-like lines (WR 2.3 and P388D1) and from rabbit leukocytes obtained by bronchoalveolar lavage (>90% macrophages). In this report, we present the complete sequence of human integrin β_7 and evidence for alternative splicing of β_7 mRNA, and we demonstrate that integrin β_7 mRNA is expressed by a variety of leukocyte lines.

MATERIALS AND METHODS

Library Screening—A 292-nucleotide partial cDNA previously obtained from Raji cells by homology PCR (17) was used as a template for the random-primed synthesis of ^{32}P-labeled probe (Multiprime DNA labeling system, Amersham Corp.). This probe was used to screen a cDNA library made from the T leukemia cell line PEER (18) and a second cDNA library made from unstimulated peripheral

* This work was supported in part by a grant from the American Lung Association of California Research Program, by Grants 1KT 71 and 1RT 338 from the University of California Tobacco-related Disease Research Program, and by Grant HL/AI33259 and Pulmonary Vascular Specialized Center for Research Grant HL19155 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M62880.

‡ An American Lung Association Edward Livingston Trudeau Scholar. To whom correspondence and reprint requests should be addressed: Lung Biology Center, UC Box 0854, San Francisco, CA 94143. Tel.: 415-821-5959; Fax: 415-282-5998.

§ Supported by a grant from the Swiss National Science Foundation.

[1] The abbreviations used are: PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); EBV, Epstein-Barr virus.

T lymphocytes obtained from a normal volunteer. Both libraries were constructed in the λgt-10 vector and were generously provided to us by Dr. D. Littman (University of California, San Francisco). In the first round, ~5 × 10^5 clones from each library were screened. Seven hybridizing clones from the PEER library (designated P1–P7) and five from the peripheral T cell library (T1–T5) were obtained. Clones P2, T2, and T3 were plaque-purified and subcloned into the pBluescript plasmid (Stratagene) for sequencing. To obtain additional clones that extended further toward the 5′-end of the complete cDNA, a 248-nucleotide fragment from the 5′-end of clone T3 was made using PCR and was used as a template for synthesis of a second probe. The second probe was used to rescreen the remaining clones from the first round; clones P7, T1, and T4 were again positive. The second probe was also used to screen ~5 × 10^5 additional PEER cell cDNA clones, and two additional clones (P8 and P9) were obtained. These clones were all plaque-purified and subcloned into pBluescript for sequencing.

DNA Sequencing—The sequence reported here was determined from all eight independent cDNA clones obtained from library screening plus two independent cDNA clones previously obtained from MoLT-4 and Raji cells by homology PCR (17). After subcloning into pBluescript, the 5′- and 3′-ends of the clones were sequenced using T7 and T3 oligonucleotide primers, T7 DNA polymerase (Promega Biotec), and the Sequenase reagent kit (United States Biochemical Corp.). Fragments obtained by *Xho*I, *Apa*I, or *Sac*I digestion of selected clones were cloned into pBluescript for internal sequencing. Additional internal sequence was obtained from the Exo/Mung deletion system (Stratagene) and with 10 internal sequencing primers (see Fig. 1). The sequence of the GC-rich region beginning at nucleotide 341 and ending at nucleotide 370 could not be determined unambiguously with T7 DNA polymerase and the standard dGTP-containing sequencing reagent mixture. Both DNA strands were sequenced with *Taq* polymerase (TaqTrack, Promega Biotec) and with T7 DNA polymerase plus a deoxyinosine triphosphate-containing sequencing reagent mixture (United States Biochemical Corp.) to confirm the sequence in this region. 92% of the cDNA sequence and 100% of the sequence of the predicted open reading frame were obtained from both strands of DNA. 99% of the cDNA sequence and 100% of the sequence of the predicted open reading frame were obtained from two or more independent clones (see Fig. 1).

Cell Culture—Mouse cell lines studied were WR 2.3 T lymphoma cells (19) and P388D1 macrophage-like cells (ATCC CCL 46). Human leukocyte lines employed were HuT 78 T cells (ATCC TIB 161), Raji Burkitt lymphoma cells (ATCC CCL 86), EBV-B6.1 EBV-transformed peripheral B cells (kind gift of Dr. S. Abrignani, Ciba/Geigy, Basel), and U-937 histiocytic lymphoma cells (ATCC CRL 1593). Peripheral T cells were purified from fresh buffy coat preparations (Irwin Memorial Blood Bank) by density gradient centrifugation (Histopaque-1077, Sigma) and nylon wool filtration (20). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal or neonatal calf serum. For stimulation experiments, cells were cultured in supplemented medium alone, in supplemented medium plus phorbol 12-myristate 13-acetate (PMA) (25 ng/ml, Calbiochem), in supplemented medium plus ionomycin (250 ng/ml, Calbiochem), and/or in supplemented medium plus both PMA and ionomycin. Cells were harvested after 1–3 days for RNA purification.

RNA Preparation—Total cellular RNA was obtained by the LiCl/urea method as follows. Cells were lysed and homogenized in 3 M LiCl, 6 M urea. After incubation for 16 h at 4 °C, lysates were centrifuged at 14,000 rpm for 30–60 min. The pellet was resuspended in HES buffer (10 mM HEPES, 1 mM EDTA, 1% SDS). After sequential phenol and chloroform extractions, RNA was precipitated with sodium acetate and ethanol and resuspended in RNase-free water. RNA concentration and purity were assessed by measurements of absorbance at 260 and 280 nm. Poly(A)$^+$ RNA, used only for Northern blot analysis of mouse cell lines, was purified by oligo(dT)-cellulose affinity chromatography (21).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Amplification of β_7 mRNA—Single-stranded cDNA was synthesized from total RNA (~7–8 μg/20-μl reaction) using modified Moloney murine leukemia virus reverse transcriptase (SuperScript, GIBCO/Bethesda Research Laboratories). Control reactions, identical except for the omission of reverse transcriptase, were performed simultaneously. Oligonucleotide primers used for amplifications were P1079F (containing the sequence of nucleotides 1079–1098 of β_7), P1289F (nucleotides 1289–1308), P2216R (reverse complement of nucleotides 2196–2216), and P2400R (reverse complement of nucleotides 2381–2400). Sequential (nested) amplifications were performed. An initial amplification (30 cycles) with P1079F and P2400R was followed by a second amplification (20 cycles) with P1289F and P2216R. Template for the initial amplification was 2 μl of cDNA/50-μl reaction. One μl of the initial amplification product/100-μl reaction was used as template for the second amplification. Amplifications were performed in 1 × *Taq* buffer (Promega Biotec) with 100 μM each dATP, dCTP, dGTP, and dTTP, a 1 pmol/μl concentration of each primer, and 0.025 unit/μl *Taq* DNA polymerase (Promega Biotec). Amplification was performed in a thermal cycler (Ericomp) as follows: 4 min at 95 °C; 20 or 30 cycles of 45 s at 95 °C, 1 min at 57 °C, and 1 min at 72 °C; and then 10 min at 72 °C. Amplification products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Northern Blot Analysis—RNA was electrophoresed through formaldehyde-agarose gels and transferred to nylon membranes (Hybond-N, Amersham Corp). Radiolabeled probe was made using the random primer method. Probes used for analyzing mouse cell RNA were synthesized from a 292-nucleotide mouse β_7 partial cDNA (17), whereas human β_7 probes were synthesized using a 1905-nucleotide fragment of clone P2 (nucleotides 857–2761). Filters were hybridized in 5 × SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 40% formamide, 20 mM Tris, pH 7.5, 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), and 10% dextran sulfate at 50 °C for ~16 h and washed in 0.5–2 × SSC and 0.1% SDS at 50 °C for 30–60 min. After washing, filters were exposed to film at −70 °C with an intensifying screen.

RESULTS

Cloning and Sequencing of Novel Integrin β Subunit cDNA from Two Human Lymphocyte Libraries—We used a novel partial cDNA obtained from Raji cells by homology PCR (17) to screen two human T lymphocyte cDNA libraries, one made from the PEER cell line and one from peripheral T cells. In two rounds of screening, we purified and sequenced four independent hybridizing clones from each library (Fig. 1).

We determined the 2767-nucleotide cDNA sequence shown

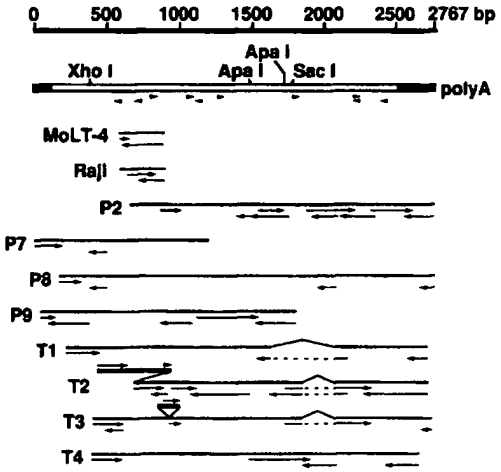


FIG. 1. **Overlapping clones used for sequence analysis.** A schematic representation of the β_7 cDNA appears immediately below the scale. The 5′- and 3′-untranslated regions are shown as *solid bars*; the 2394-nucleotide open reading frame is shown as an *open bar*. Recognition sites for *Xho*I, *Apa*I, and *Sac*I restriction endonucleases (used to generate fragments for internal sequencing) are shown. *Arrowheads* represent oligonucleotide primers used to obtain internal sequence. Clones labeled *MoLT-4* and *Raji* were obtained from lymphoma cell cDNA using homology PCR, as reported previously (17). A probe made using the Raji clone was used to screen two cDNA libraries, and clones P2 (from the PEER cell library) and clones T2 and T3 (from the peripheral T cell library) were obtained. Clones P7–P9 (PEER) and clones T1 and T4 (peripheral T cell) were obtained using a probe derived from the 5′-end of clone T3. The direction and extent of sequencing of each clone are shown by *arrows*. Clones T2 and T3 had additional sequence, shown as *thick bars*, not present in other clones. Clones T1–T3 are missing portions of the sequence that are present in other clones, as shown.

in Fig. 2 from these eight overlapping clones plus the partial cDNAs previously obtained by PCR. The sequence contains a single large open reading frame predicted to encode a 798-amino acid protein precursor. We obtained the sequence of 113 nucleotides of the 5′-untranslated region and 260 nucleotides of the 3′-untranslated region. A standard polyadenylation signal (AATAAA) is present near the end of the 3′-untranslated region, just before the poly(dA) tail.

We have designated the protein encoded by this novel cDNA as integrin β_7 because of the similarity of this protein to the six previously sequenced mammalian integrin β subunits. The β_7 deduced amino acid sequence is shown in Fig. 2 and is compared with other β subunit sequences in Fig. 3. β_7 is 46% identical to β_2, 43% identical to β_1, 38% identical to β_3 and β_5, and 37% identical to β_6. If the unique large cytoplasmic portion of β_4 is excluded from the analysis, β_7 is 32% identical to β_4. The general domain structure of β_7 also appears to be quite similar to the structure of the other β subunits. Like other integrin β subunits, β_7 contains a single putative transmembrane domain (see Fig. 3). By analogy with other β subunits, the 48 amino acid residues on the carboxyl-terminal end of β_7 are predicted to compose the cytoplasmic domain. Overall, the β_7 cytoplasmic domain is similar to the cytoplasmic domains of other β subunits. However, β_7 does contain a unique five-amino acid carboxyl-terminal extension that distinguishes it from even its closest relatives, β_2 and β_1.

The amino-terminal 726 amino acid residues are predicted to make up the signal peptide and the extracellular portion of β_7. As expected, the putative signal peptide contains a short stretch of strongly hydrophobic amino acid residues. Cleavage of the signal peptide at the predicted site (22) would produce a mature β_7 protein with a unique amino-terminal extension of ~20 amino acid residues that is not present in other β subunits (Fig. 3). The region of the extracellular domain beginning at residue 151 and extending to residue 286 is especially highly conserved; β_7 is 49–66% identical to the previously sequenced β subunits in this region. There are eight potential *N*-glycosylation sites in the extracellular portion of β_7. If all eight sites are occupied with carbohydrate side chains with average M_r of 2500, the predicted molecular weight of mature β_7 would be 105,000. There are 56 cysteine residues that are conserved in the extracellular portions of

```
1       AGCCCAGAGAGAAAGTCTGACTTGCCCCACAGCCAGTGAGTGACTGCAGCAGCACCAGAATCTGGTCTGTTTCCTGTTTGGCTCTTCTACCACTACGGCTTGGGATCTCGGGC
114     ATGGTGGCTTTGCCAATGGTCCTTGTTTTGCTGCTGGTCCTGAGCAGAGGTGAGAGTGAATTGGACGCCAAGATCCCATCCACAGGGGATGCCACAGAATGGCGGAATCCTCACCTGTCC
        m v a l p m v l v l l l v l s r g e s E L D A K I P S T G D A T E W R N P H L S   40
234     ATGCTGGGGTCCTGCCAGCCAGCCCCCTCCTGCCAGAAGTGCATCCTCTCACACCCCAGCTGTGCATGGTGCAAGCAACTGAACTTCACCGCGTCGGGAGAGGCGGAGGCGCGGCGCTGC
        M L G S C Q P A P S C Q K C I L S H P S C A W C K Q L N F T A S G E A E A R R C   80
354     GCCCGACGAGAGGAGCTGCTGGCTCGAGGCTGCCCGCTGGAGGAGCTGGAGGAGCCCCGCGGCCAGCAGGAGGTGCTGCAGGACCAGCCGCTCAGCCAGGGCGCCCGCGGAGAGGGTGCC
        A R R E E L L A R G C P L E E L E E P R G Q Q E V L Q D Q P L S Q G A R G E G A   120
474     ACCCAGCTGGCGCCGCAGCGGGTCCGGGTCACGCTGCGGCCTGGGGAGCCCCAGCAGCTCCAGGTCCGCTTCCTTCGTGCTGAGGGATACCCGGTGGACCTGTACTACCTTATGGACCTG
        T Q L A P Q R V R V T L R P G E P Q Q L Q V R F L R A E G Y P V D L Y Y L M D L   160
594     AGCTACTCCATGAAGGACGACCTGGAACGCGTGCGCCAGCTCGGGCACGCTCTGCTGGTCCGGCTGCAGGAAGTCACCCATTCTGTGCGCATTGGTTTTGGTTCCTTTGTGGACAAAACG
        S Y S M K D D L E R V R Q L G H A L L V R L Q E V T H S V R I G F G S F V D K T   200
714     GTGCTGCCCTTTGTGAGCACAGTACCCTCCAAACTGCGCCCACCCCTGCCCCACCCGGCTGGAGCGCTGCCAGTCACCATTCAGCTTTCACCATGTGCTGTCCCTGACGGGGGACGCACAA
        V L P F V S T V P S K L R H P C P T R L E R C Q S P F S F H H V L S L T G D A Q   240
834     GCCTTCGAGCGGGAGGTGGGGCGCCAGAGTGTGTCCGGCAATCTGGACTCGCCTGAAGGTGGCTTCGATGCCATTCTGCAGGCTGCACTCTGCCAGGAGCAGATTGGCTGGAGAAATGTG
        A F E R E V G R Q S V S G N L D S P E G G F D A I L Q A A L C Q E Q I G W R N V   280
954     TCCCGGCTGCTGGTGTTCACTTCAGACGACACATTCCATACAGCTGGGGACGGGAAGTTGGGCGGCATTTTCATGCCCAGTGATGGGCACTGCCACTTGGACAGCAATGGCCTCTACAGT
        S R L L V F T S D D T F H T A G D G K L G G I F M P S D G H C H L D S N G L Y S   320
1074    CGCAGCACAGAGTTTGACTACCCTTCTGTGGGTCAGGTAGCCCAGGCCCTCTCTGCAGCAAATATCCAGCCCATCTTTGCTGTCACCAGTGCCGCACTGCCTGTCTACCAGGAGCTGAGT
        R S T E F D Y P S V G Q V A Q A L S A A N I Q P I F A V T S A A L P V Y Q E L S   360
1194    AAACTGATTCCTAAGTCTGCAGTTGGGGAGCTGAGTGAGGACTCCAGCAACGTGGTACAGCTCATCATGGATGCTTATAATAGCCTGTCTTCCACTGTGACCCTTGAACACTCTTCACTC
        K L I P K S A V G E L S E D S S N V V Q L I M D A Y N S L S S T V T L E H S S L   400
1314    CCTCCTGGGGTCCACATTTCTTACGAATCCCAGTGTGAGGGTCCTGAGAAGAGGGAGGGTAAGGCTGAGGATCGAGGACAGTGCAACCACGTCCGAATCAACCAGACGGTGACTTTCTGG
        P P G V H I S Y E S Q C E G P E K R E G K A E D R G Q C N H V R I N Q T V T F W   440
1434    GTTTCTCTCCAAGCCACCCACTGCCTCCCAGAGCCCCATCTCCTGAGGCTCCGGGCCCTTGGCTTCTCAGAGGAGCTGATTGTGGAGTTGCACACGCTGTGTGACTGTAATTGCAGTGAC
        V S L Q A T H C L P E P H L L R L R A L G F S E E L I V E L H T L C D C N C S D   480
1554    ACCCAGCCCCAGGCTCCCCACTGCAGTGATGGCCAGGGACACCTACAATGTGGTGTATGCAGCTGTGCCCCTGGCCGCCTAGGTCGGCTCTGTGAGTGCTCTGTGGCAGAGCTGTCCTCC
        T Q P Q A P H C S D G Q G H L Q C G V C S C A P G R L G R L C E C S V A E L S S   520
1674    CCAGACCTGGAATCTGGGTGCCGGGCTCCCAATGGCACAGGGCCCCTGTGCAGTGGAAAGGGTCACTGTCAATGTGGACGCTGCAGCTGCAGTGGACAGAGCTCTGGGCATCTGTGCGAG
        P D L E S G C R A P N G T G P L C S G K G H C Q C G R C S C S G Q S S G H L C E   560
1794    TGTGACGATGCCAGCTGTGAGCGACATGAGGGCATCCTCTGCGGAGGCTTTGGTCGCTGCCAATGTGGAGTATGTCACTGTCATGCCAACCGCACGGGCAGAGCATGCGAATGCAGTGGG
        C D D A S C E R H E G I L C G G F G R C Q C G V C H C H A N R T G R A C E C S G   600
1914    GACATGGACAGTTGCATCAGTCCCGAGGGAGGGCTCTGCAGTGGGCATGGACGCTGCAAATGCAACCGCTGCCAGTGCTTGGACGGCTACTATGGTGCTCATGCGACCAATGCCCAGGC
        D M D S C I S P E G G L C S G H G R C K C N R C Q C L D G Y Y G A L C D Q C P G   640
2034    TGCAAGACACCATGCGAGAGACACCGGGACTGTGCAGAGTGTGGGGCCTTCAGGACTGGCCCACTGGCCACCAACTGCAGTACAGCTTGTGCCCATACCAATGTGACCCTGGCCTTGGCC
        C K T P C E R H R D C A E C G A F R T G P L A T N C S T A C A H T N V T L A L A   680
2154    CCTATCTTGGATGATGGCTGGTGCAAAGAGCGGACCCTGGACAACCAGCTGTTCTTCTTGGTGGAGGATGACGCCAGAGGCACGGTCGTGCTCAGAGTGAGACCCCAAGAAAAGGGA
        P I L D D G W C K E R T L D N Q L F F F L V E D D A R G T V V L R V R P Q E K G   720
2274    GCAGACCACACGCAGGCCATTGTGCTGGGCTGCGTAGGGGGCATCGTGGCAGTGGGGCTGGGGCTGGTCCTGGCTTACCGGCTCTCGGTGGAAATCTATGACCGCCGGGAATACAGTCGC
        A D H T Q A I V L G C V G G I V A V G L G L V L A Y R L S V E I Y D R R E Y S R   760
2394    TTTGAGAAGGAGCAGCAACAACTCAACTGGAAGCAGGACAGTAATCCTCTCTACAAAAGTGCCATCACGACCACCATCAATCCTCGCTTTCAAGAGGCAGACAGTCCCACTCTCTGAAGG
        F E K E Q Q Q L N W K Q D S N P L Y K S A I T T T I N P R F Q E A D S P T L   798
2514    AGGGAGGGACACTTACCCAAGGCTCTTCTCCTTGGAGGACAGTGGGAACTGGAGGGTGAGAGGAAGGGTGGGTCTGTAAGACCTTGGTAGGGGACTAATTCACTGGCGAGGTGCGGCCAC
2634    CACCCTACTTCATTTTCAGAGTGACACCCAAGAGGGCTGCTTCCCATGCCTGCAACCTTGCATCCATCTGGGCTACCCCACCCAAGTATACAATAAAGTCTTACCTCAGAAAAAAAAAAA
2754    AAAAAAAAAAAAA 2767
```

FIG. 2. **Human integrin β_7 cDNA sequence and deduced amino acid sequence.** The amino acid sequence is shown in one-letter code below the first nucleotide of each codon. The putative signal peptide (amino acid residues 1–19) is shown in *lower-case letters*, and the putative transmembrane domain (residues 727–750) by a *solid underline*. *Underlined* asparagine residues (*N*) are potential sites for *N*-glycosylation. Nucleotides 1616–2059 (*dotted overline*) are not present in clone T1. The partial β_7 clone previously obtained using homology PCR (nucleotides 593–884) is indicated by a *dashed overline*.

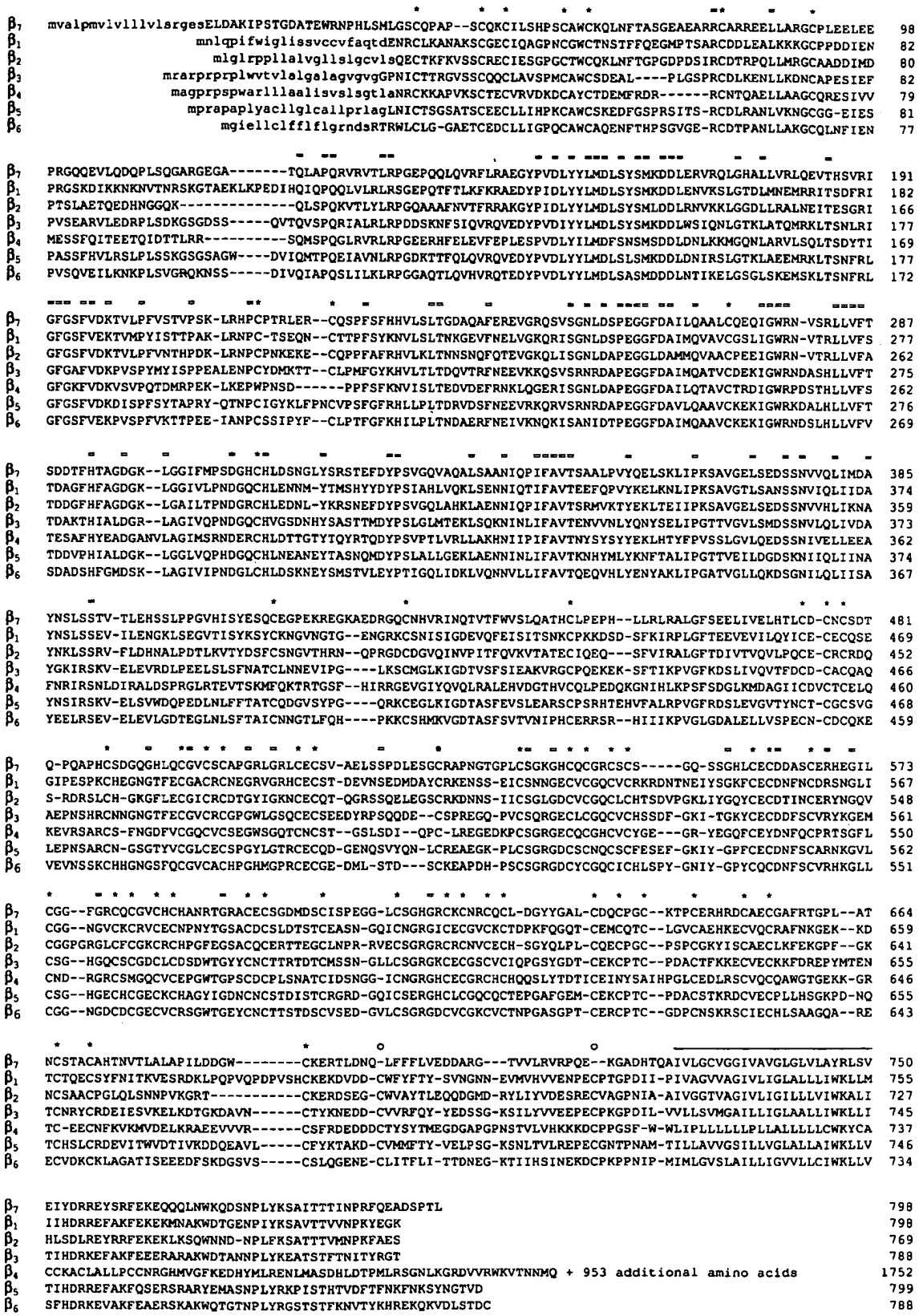


FIG. 3. **Alignment of β_7 deduced amino acid sequence with six previously reported human integrin β subunit sequences.** The deduced amino acid sequences of β_7 and β_1–β_6 (7–16) are shown in one-letter code. Signal peptides are shown in *lower-case letters*. Signal peptide cleavage sites have been previously determined by direct protein sequencing of β_1–β_5; the β_6 and β_7 cleavage sites were predicted according to von Heijne (22). The putative transmembrane domains are indicated by the *solid overline*. Most of the cytoplasmic portion of β_4 is

A

```
                192              273              501              576              649
                 |                |                |                |                |
β7    THSVRIGFGSFVDK ... QAALCQEQIGWRN ... CGVCSCAPGRLG ... GILCGGFGRCQC ... CERHRDCAEC

                183              263              490              570              644
                 |                |                |                |                |
β1    TSDFRIGFGSFVEK ... QVAVCGSLIGWRN ... CGACRCNEGRVG ... GLICGGNGVCKC ... CAEHKECVQC

                                 260                               564              638
                                  |                                |                |
β3    TSNLRIGFGAFVDK ... QATVCDEKIGWRN ... CGVCRCGPGWLG ... GEMCSGHGQCSC ... CTFKKECVEC
```

B

```
                  *        *    * * * *          *  *
Repeat I      CSDGQGHL-QC---GVCS-CAP-----GRLGRL---CE--CSVAELSSPDLESG
Repeat II     CRAPNGTGPLCSGKGHCQ-CGRCSCSGQSSGHL---CE--CDDAS
Repeat III    CERHEGIL--CGGFGRCQ-CGVCHCHANRTGRA---CE--CSGDMDS
Repeat IV     CISPEGGL--CSGHGRCK-CNRCQCLDGYYGAL---CDQ-CPG
Repeat V      CKTP------CERH---RDCAECG--AFRTGPLATNCSTAC
                                       ↓
Alternative   CSDGQGHL-QC---GVCRDCAECG--AFRTGPLATNCSTAC
splice
```

FIG. 4. **Positions of β_7 putative splice sites.** *A*, Comparison of five β_7 putative exon boundaries with known β_1 and β_3 exon boundaries. Fragments of the deduced amino acid sequences of integrins β_7, β_1, and β_3 are shown; the alignment and numbering system are derived from Fig. 3. Positions of exon boundaries are indicated by *vertical lines*. Positions of putative exon boundaries of β_7 were determined from alternatively spliced clones T2–T4 (Fig. 1). Clones T2 and T3 have putative intron sequences present at positions corresponding to amino acid residues 192 and 273, respectively. Clones T2 and T3 both have frame-shifting deletions of nucleotides 1840–2059 (corresponding to amino acid residues 576–649). Clone T1 has an in-frame deletion of 444 nucleotides (nucleotides 1616–2059) encoding amino acids 501–649. Selected exon boundaries for β_1 and β_3 are shown for comparison (23, 24). *B*, the typical form of β_7 is predicted to contain a total of five cysteine-rich repeats in the extracellular domain, as do previously sequenced β subunits. The three middle repeats are complete repeats containing all 8 cysteine residues; the first and fifth repeats are incomplete. Translation of mRNA containing the in-frame deletion identified in clone T1 would be predicted to result in a smaller protein containing the first portion of the first repeat and the terminal portion of the fifth repeat, joined at the arginine residue (*R*) indicated by the *arrow*. Both sequences begin at amino acid residue 488. Gaps (indicated by *dashes*) were introduced to maximize alignment. The positions of conserved cysteine residues are indicated by *asterisks*.

β_1–β_3, β_5, and β_6. All but eight are conserved in β_4. In β_7, 54 of these 56 cysteine residues are conserved. All previously sequenced β subunits have one pair of cysteine residues located very near the transmembrane domain; these 2 cysteine residues are not present in β_7 (Fig. 3).

Alternatively Spliced β_7 cDNA Clones—Three of the four clones isolated from the peripheral T cell cDNA library apparently represent alternatively spliced mRNAs (Fig. 1). Clone T1 has an in-frame deletion of bases 1616–2059; the putative exon boundaries deduced from this deletion correspond to previously reported β_1 and β_3 exon boundaries (23, 24) (Fig. 4*A*). If mRNA with this deletion were translated, the predicted protein product would be a second form of β_7 that is missing 148 amino acid residues that compose most of the cysteine-rich region of the extracellular domain (Fig. 4*B*) (see "Discussion"). The predicted molecular weight of this form of β_7, assuming all six remaining potential glycosylation sites are occupied, would be ~85,000.

Clones T2 and T3 each appear to represent incompletely processed mRNAs containing residual introns. The position of the clone T2 putative intron is analogous to the position of an intron found in the integrin β_1 gene, whereas the position of the clone T3 putative intron is analogous to the position of introns found in both the β_1 and β_3 genes (23, 24) (Fig. 4*A*). The sequences of the 3′-ends of both putative introns are in generally good agreement with the acceptor site consensus sequence, 5′-Pyr$_n$*X*CAG$\underset{T}{G}$-3′, where Pyr is a pyrimidine, *X* is A, C, G, or T, and underlined residues are invariant (25). The clone T2 putative acceptor site is 5′-TTCCTCTC-CTCTCCCAGGT-3′, and the clone T3 putative acceptor site is 5′-CCCCCACTGTAACTCAGGA-3′. Clones T2 and T3 also contain identical frame-shifting deletions of 220 bases. The 3′-end (base 2059) of the deleted fragment coincides with the 3′-end of the larger deleted fragment of clone T1 (Fig. 1). The 5′-end (base 1840) of the clone T2/clone T3 deletion is analogous to exon boundaries in the β_1 and β_3 genes.

Identification of β_7 Splicing Variants Using RT-PCR—We used the RT-PCR technique to detect alternative splicing of β_7 in lymphocytes. Two pairs of β_7-specific oligonucleotide primers were used for nested amplification of cDNA. Amplification of cDNAs that have no deleted exons (for example, clone P2) results in a 928-bp product, whereas amplification of cDNAs containing the 444-nucleotide deletion (clone T1) results in a 484-bp product (Fig. 5). The 484-bp cDNA is amplified significantly more efficiently than the larger 928-bp product (data not shown). RT-PCR of unstimulated peripheral T cell RNA resulted in a single cDNA product of 484

omitted. Sequences are numbered beginning with the methionine residue that begins each signal peptide. *Dashes* indicate gaps introduced to maximize alignment. Cysteine residues that are conserved in β_7 and β_1–β_3, β_5, and β_6 are indicated by *asterisks*. The 2 cysteine residues present in all β subunits except β_7 are indicated by *open circles*. Other residues conserved in all β subunits are denoted by *equal signs*.

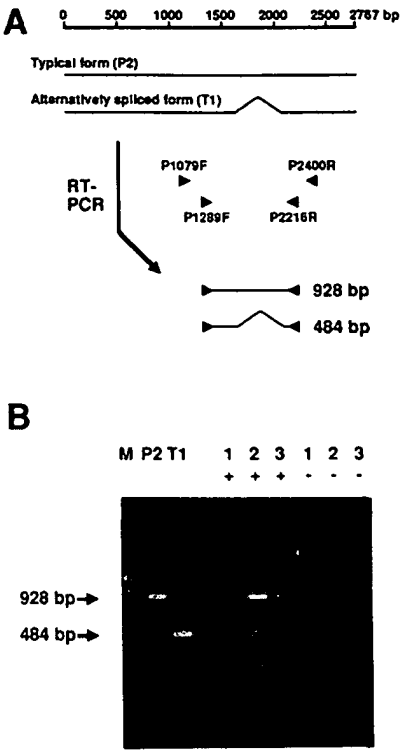


FIG. 5. **Analysis of β_7 splicing variants by RT-PCR.** *A*, schematic representations of two forms of β_7 mRNA are shown. The typical form (represented, for example, by cDNA clone P2) is larger than the alternatively spliced form (represented by clone T1), which has a 444-bp in-frame deletion. After reverse transcription of cellular RNA, cDNA was sequentially amplified with nested primer pairs (P1079F-P2400R, then P1289F-P2216R) by PCR. Amplification of the typical form of β_7 is predicted to produce a 928-bp cDNA product, whereas amplification of the alternatively spliced cDNA is predicted to produce a 484-bp product. *B*, agarose gel electrophoresis of RT-PCR products. *Lane M* contains ϕX-*Hae*III digest size markers. As a positive control, cloned cDNAs representing the typical form of β_7 (P2) and the alternatively spliced form of β_7 (T1) were amplified using the P1289F-P2216R primers. Cellular RNAs from unstimulated peripheral T cells (1), PMA-stimulated peripheral T cells (2), and PMA-stimulated HuT 78 cells (3) were used for RT-PCR. Single-stranded cDNA was synthesized using reverse transcriptase (+); parallel control reactions were performed without reverse transcriptase (−). The cDNA was amplified by PCR and visualized on an ethidium bromide-stained agarose gel.

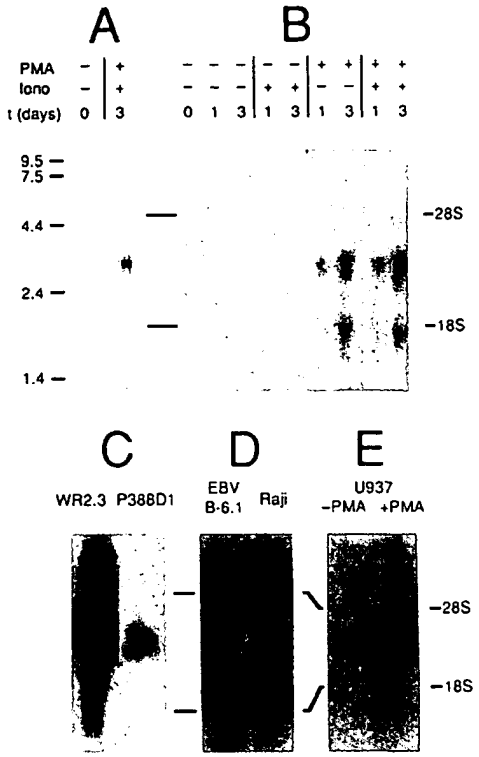


FIG. 6. **Identification of β_7 mRNA in leukocytes by Northern blot analysis.** Stimulated and unstimulated human and mouse leukocytes were probed for β_7 mRNA. *A*, analysis of total RNA (25 μg/lane) obtained from human peripheral T cells immediately after purification and after 3 days of incubation with PMA (25 ng/ml) and ionomycin (*Iono*) (250 ng/ml). The positions and sizes (in kilobases) of RNA markers are indicated. *B*, analysis of total RNA (25 μg/lane) obtained from unstimulated HuT 78 cells at 0, 1, and 3 days and from stimulated cells at 1 and 3 days. Cells were stimulated with ionomycin alone, with PMA alone, or with both ionomycin and PMA. *C*, analysis of poly(A)$^+$ RNA (10 μg/lane) from unstimulated mouse WR 2.3 T cells and P388D1 macrophage-like cells. *D*, analysis of total RNA from unstimulated EBV-B6.1 cells (30 μg) and Raji cells (20 μg). *E*, analysis of total RNA (50 μg/lane) obtained from U-937 cells cultured for 2 days in the absence or presence of PMA (25 ng/ml). *C–E* represent shorter gel runs, and these autoradiographs are enlarged ~2.5 times compared with *A* and *B*. Positions of the 28 S and 18 S ribosomal RNA bands are indicated for each gel. In PMA stimulation experiments (*A*, *B*, and *E*), ethidium bromide staining was used to verify that all lanes contained similar amounts of RNA. A human β_7 probe was used with human RNA (*A*, *B*, *D*, and *E*), and a mouse β_7 probe was used with mouse RNA (*C*).

bp (Fig. 5). The 928-bp product was not detected in these cells (see "Discussion"). In contrast, RT-PCR of RNA from PMA-stimulated peripheral T cells or HuT 78 T lymphoma cells yielded both 928- and 484-bp products. Restriction analysis confirmed that the 928-bp products amplified by RT-PCR are authentic typical β_7 cDNAs and that the 484-bp products are authentic alternatively spliced β_7 cDNAs (data not shown). The nature of a third RT-PCR product of even smaller size (~270 bp) obtained from PMA-stimulated peripheral T cell RNA is unclear. This product could represent another β_7 splicing variant or a PCR artifact.

Detection of β_7 mRNA by Northern Blotting—We used Northern blot analysis to study the distribution and regulation of β_7 mRNA. β_7 mRNA was not detected in unstimulated peripheral T cells, but β_7 mRNA of ~3.2 kilobases was easily detectable after 3 days of stimulation with PMA plus ionomycin (Fig. 6*A*). The regulation of β_7 mRNA was studied in more detail using HuT 78 T lymphoma cells (Fig. 6*B*). These cells also have a marked increase in β_7 mRNA after stimulation with PMA plus ionomycin. In HuT 78 cells, stimulation with PMA alone is sufficient to induce a marked increase in β_7 mRNA, whereas ionomycin alone has no detectable effect. The effect of PMA is detectable within 1 day and is substantially greater by 3 days.

We also detected β_7 mRNA in unstimulated mouse WR 2.3 T lymphoma and P388D1 macrophage-like cell lines and in unstimulated human EBV-B6.1 EBV-transformed B cells and Raji Burkitt lymphoma cells (Fig. 6, *C* and *D*). After stimulation with PMA, U-937 cells differentiate from promonocyte-like cells to macrophage-like cells (26); this is accompanied by a marked increase in β_7 mRNA (Fig. 6*E*). Alternatively spliced β_7 mRNAs were not detected in any of the Northern blot experiments (see "Discussion"). β_7 mRNA was not detectable in the six nonleukocyte cell lines we studied (A549, Calu-3, and SK-MES-1 lung carcinoma cell lines; FG-2 pancreatic carcinoma cells; HeLa cervical carcinoma cells; and HT-1080 fibrosarcoma cells) (data not shown).

DISCUSSION

This report presents the amino acid sequence, deduced from human lymphocyte cDNA, of an integrin β subunit that we designate as β_7 (Fig. 2). Comparison of the novel protein sequence with the six previously reported human integrin β subunit sequences (Fig. 3) clearly establishes that the protein is a member of the integrin β subunit family. The β_7 amino acid sequence is 32–46% identical to the sequences of integrins β_1–β_6. β_7 is most similar to β_2, the only previously sequenced integrin β subunit thought to be restricted to leukocytes. Like other β subunits, β_7 is predicted to contain a large (~700-amino acid) extracellular portion, a transmembrane domain, and a cytoplasmic domain. The β_7 sequence is very similar (49–66% identity) to other β subunit sequences in a 136-amino acid region of the extracellular domain (amino acid residues 151–286). Cross-linking studies indicate that this region of β_3 is in close proximity to ligand during binding (27, 28). There are also several unique features of β_7 that distinguish it from β_1–β_6. The extracellular domain of β_7 apparently contains an amino-terminal extension of ~20 amino acid residues not present in β_1–β_6. The significance of this extension is unclear since the function of the amino-terminal region of integrin β subunits is not known. The region of the extracellular portion of β_7 near the transmembrane domain also differs from other integrin β subunits: the 2 conserved cysteine residues nearest the transmembrane domain, which are present in all other β subunits, are missing from β_7. All other 54 conserved cysteine residues are preserved in β_7. This difference in cysteine content is likely to affect the formation of disulfide bonds that help determine β subunit folding. Finally, the β_7 cytoplasmic domain has a 5-amino acid residue carboxyl-terminal extension when compared to β_1–β_3. There is no sequence similarity between this extension and the somewhat longer extensions of β_5 and β_6. It is possible that these distinctive carboxyl-terminal extensions help direct subunit-specific interactions with cytoskeletal elements, signal transduction pathways, or cytoplasmic regulatory factors.

Analysis of variant β_7 cDNA clones obtained during library screening suggests that β_7 mRNA may be alternatively spliced in peripheral T cells. Clone T4 (from the peripheral T cell library) and clones P2 and P8 (from the PEER library) all correspond to mRNAs encoding a typical integrin β subunit containing a large cysteine-rich region (Figs. 1 and 2). Clone T1 (from the peripheral T cell library) has an in-frame deletion of 444 nucleotides (bases 1616–2059) encoding 148 amino acid residues from the cysteine-rich region. Two other cDNA clones (T2 and T3) obtained from the same library apparently represent partially processed mRNAs with related exon deletions. These mRNAs are apparently not mature since each contains one putative intron; both also have identical frame-shifting deletions of bases 1840–2059. These intermediary forms of β_7 mRNA are presumably destined to become mature alternatively spliced mRNAs, not mature typical mRNAs, since exons have already been deleted. We hypothesize that mature alternatively spliced β_7 mRNAs are formed by the deletion of one exon (bases 1840–2059) followed by the deletion of a second adjacent exon (bases 1616–1839), although alternative explanations are possible. Of note, the five β_7 putative exon boundaries deduced from variant clones all correspond to known exon boundaries in other integrin β subunit genes (Fig. 4*A*).

We were able to confirm that alternatively spliced β_7 mRNA is present in unstimulated peripheral T cells using RT-PCR (Fig. 5), despite the fact that there is apparently very little β_7 mRNA in these cells (Fig. 6*A*). The typical form of β_7 mRNA is probably present in very small amounts in unstimulated T cells; it was represented in the peripheral T cell cDNA library (clone T4), but was not detected by RT-PCR, perhaps because this larger form is amplified less efficiently than the smaller alternatively spliced form (data not shown). In PMA-stimulated cells, which contain more β_7 mRNA, both the larger, typical form and the smaller, alternatively spliced forms were detected by RT-PCR (Fig. 5*B*). However, only a single form of β_7 mRNA was detected by Northern blotting (Fig. 6). It seems most likely that only the typical form of β_7 mRNA was detected by Northern blotting and that the alternatively spliced form of β_7 mRNA, present only in small amounts, could be detected only by the more sensitive RT-PCR method.

Our observations about alternative splicing are preliminary. There must be some doubt about the importance of these observations since alternatively spliced β_7 mRNA is probably present in relatively small amounts in the cells we studied. More important, we have no data that allow us to determine whether two different forms of the β_7 protein are actually expressed. Nonetheless, it is intriguing to speculate that integrin β_7 may exist in two forms as a result of alternative splicing in some cells. The typical form of β_7 contains five repeats of a cysteine-rich motif in the extracellular domain. In the smaller protein predicted to result from alternative splicing, most of the cysteine-rich region is deleted (Fig. 4*B*). Since the role of the cysteine repeats in β subunit assembly and function is unknown, it is difficult to predict the effect of this deletion. However, the protein that may result from alternative splicing would still contain intact transmembrane and cytoplasmic domains and the most highly conserved portion of the extracellular domain, believed to be necessary for integrin function. It is therefore conceivable that the alternative form of the β_7 protein is functional. There is now evidence that alternative splicing of adhesion molecules is a relatively common phenomenon. Alternatively spliced forms of integrins β_3 and β_4 have been identified (11–13, 29). These previously reported cases of alternative splicing are predicted to affect only the cytoplasmic portions of integrin β subunits. There is evidence suggesting that extracellular domains of integrin α subunits (30, 31) and of non-integrin adhesion molecules including GMP-140 and NCAM (32–34) may be modified by alternative splicing. The alternative splicing of GMP-140 may be similar to the alternative splicing of β_7 in that it results in the deletion of a portion of a repeated region of the extracellular domain believed to be remote from the ligand-binding site. The functional significance of alternative splicing of adhesion molecules is not yet known.

By Northern blotting, we found that stimulation of peripheral T cells or HuT 78 T lymphoma cells with PMA plus ionomycin caused a marked increase in β_7 mRNA levels (Fig. 6, *A* and *B*). This effect is apparently due primarily or exclusively to PMA; ionomycin alone had no detectable effect. Not all T cell lines require stimulation to express easily detectable levels of β_7 mRNA. We were able to detect β_7 mRNA in some unstimulated T cell, B cell, and macrophage-like lines (Fig. 6, *C* and *D*). In contrast, we have been unable to detect β_7 mRNA in a variety of nonleukocyte cell lines by Northern blotting (data not shown). Using homology PCR, we have been able to identify β_7 in leukocytes from three different species, but not in epithelial and endothelial cells from five species (17).[2] These data suggest that β_7, like its closest known relative, β_2, may be restricted to leukocytes. Northern blot analysis demonstrates that the β_7 mRNA is ~3.2 kilobases in length. The 2767-nucleotide cDNA sequence we obtained includes 113 nucleotides of the 5′-untranslated region, the

[2] D. J. Erle, C. Rüegg, D. Sheppard, and Robert Pytela, unpublished observations.

entire open reading frame, and the 3′-untranslated region including the poly(dA) tail. It is likely that the discrepancy between the apparent mRNA size and the length of our sequence indicates that the sequence we obtained does not extend to the beginning of the 5′-untranslated region.

Other investigators have described integrin β subunits that are expressed in leukocytes and that differ immunologically from previously sequenced β subunits. The murine lymphocyte β subunit β_P was identified as an alternative partner for α_4 (5, 6). The $\alpha_4\beta_P$ complex is present on the surface of certain T cells and has a role in lymphocyte homing to Peyer's patches. Integrin $\alpha_4\beta_P$ was not detected on any of the three B cell lines tested. However, it is not possible to conclude that β_P is not expressed on B cells since β_P could, in theory, associate with an α subunit other than α_4 in some cells. The apparent molecular weight of β_P on reducing SDS gels is 130,000 (5, 6), which is substantially greater than the predicted β_7 M_r of 105,000. However, this difference could be attributable to the presence of *O*-linked carbohydrates, to differences in glycosylation between species, or to anomalous migration of the protein on SDS gels. Additional data will be required to determine whether β_7 is the human homolog of mouse β_P. The "leukocyte response integrin" is expressed on neutrophils; the identity of its α and β subunits remains unknown (35). The smaller subunit (presumably the β subunit) has an apparent M_r of ~110,000, which is very close to the predicted β_7 molecular weight. There are no published data about the distribution of the leukocyte response integrin on lymphocytes or macrophages, and we have not determined whether β_7 is present on neutrophils. The relationship of β_7 to the leukocyte response integrin remains to be defined.

Each integrin β_7 molecule presumably can combine with an integrin α subunit to form a heterodimeric cell adhesion molecule. Leukocytes express a wide variety of known α subunits that may serve as β_7 partners. Since the β subunit does play a role in determining ligand specificity, β_7 integrins are likely to have distinctive properties. Alternative splicing of β_7 may affect functional features of the receptor complex, like subunit association, ligand specificity, or ligand binding affinity. Since leukocyte expression of β_7 mRNA can be markedly induced by PMA, β_7 may be regulated by a variety of physiological stimuli during immune and/or inflammatory processes.

Note Added in Proof—After this report was submitted for publication, we learned of another report (36) of a human integrin β_7 sequence that differs by 9 amino acid residues from the sequence reported here.

REFERENCES

1. Hynes, R. O. (1987) *Cell* **48**, 549–554
2. Kishimoto, T. K., Larson, R. S., Corbi, A. L., Dustin, M. L., Staunton, D. E., and Springer, T. A. (1989) *Adv. Immunol.* **46**, 149–182
3. Takada, Y., Strominger, J. L., and Hemler, M. E. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3239–3243
4. Springer, T. A. (1990) *Nature* **346**, 425–434
5. Holzmann, B., and Weissman, I. L. (1989) *EMBO J.* **8**, 1735–1741
6. Holzmann, B., McIntyre, B. W., and Weissmann, I. L. (1989) *Cell* **56**, 37–46
7. Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D., and Ruoslahti, E. (1987) *J. Cell Biol.* **105**, 1183–1190
8. Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M., and Springer, T. A. (1987) *Cell* **48**, 681–690
9. Law, S. L., Gagnon, J., Hildreth, J. E., Wells, C. E., Willis, A. C., and Wong, A. J. (1987) *EMBO J.* **6**, 915–919
10. Fitzgerald, L. A., Steiner, B., Rall, S. C. J., Lo, S. S., and Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 3936–3939
11. Suzuki, S., and Naitoh, Y. (1990) *EMBO J.* **9**, 757–763
12. Hogervorst, F., Kuikman, I., von dem Borne, A. E. G. K., and Sonnenberg, A. (1990) *EMBO J.* **9**, 765–770
13. Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M., and Quaranta, V. (1990) *J. Cell Biol.* **111**, 1593–1604
14. Ramaswamy, H., and Hemler, M. E. (1990) *EMBO J.* **9**, 1561–1568
15. Suzuki, S., Huang, Z.-S., and Tanihara, H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5354–5358
16. Sheppard, D., Rozzo, C., Starr, L., Quaranta, V., Erle, D. J., and Pytela, R. (1990) *J. Biol. Chem.* **265**, 11502–11507
17. Erle, D. J., Sheppard, D., Breuss, J., Rüegg, C., and Pytela, R. (1991) *Am. J. Resp. Cell Mol. Biol.*, in press
18. Littman, D. R., Newton, M., Crommie, D., Ang, S. L., Seidman, J. G., Gettner, S. N., and Weiss, A. (1987) *Nature* **326**, 85–88
19. Cardarelli, P. M., and Pierschbacher, M. D. (1987) *J. Cell Biol.* **105**, 499–506
20. Greaves, M. F., Janossay, G., and Curtis, P. (1976) in *In Vitro Methods in Cell-mediated and Tumor Immunity* (Bloom, B. R., and David, J. R., eds) pp. 231–240, Academic Press, New York
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690
23. Zimrin, A. B., Gidwitz, S., Lord, S., Schwartz, E., Bennett, J. S., White, G. C. I., and Poncz, M. (1990) *J. Biol. Chem.* **265**, 8590–8595
24. Lanza, F., Kieffer, N., Phillips, D. R., and Fitzgerald, L. A. (1990) *J. Biol. Chem.* **265**, 18098–18103
25. Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383
26. Radzun, H. J., Parwaresch, M. R., Sundstrom, C., Nilsson, K., and Eissner, M. (1983) *Int. J. Cancer* **31**, 181–186
27. D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C., and Plow, E. F. (1988) *Science* **242**, 91–93
28. Smith, J. W., and Cheresh, D. A. (1988) *J. Biol. Chem.* **263**, 18726–18731
29. van Kuppevelt, T. H., Languino, L. R., Gailit, J. O., Suzuki, S., and Ruoslahti, E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5415–5418
30. Bray, P. F., Leung, C. S., and Shuman, M. A. (1990) *J. Biol. Chem.* **265**, 9587–9590
31. Brown, N. H., King, D. L., Wilcox, M., and Kafatos, F. C. (1989) *Cell* **59**, 185–195
32. Johnston, G. I., Cook, R. G., and McEver, R. P. (1989) *Cell* **56**, 1033–1044
33. Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R., and Edelman, G. M. (1987) *Science* **236**, 799–806
34. Santoni, M. J., Barthels, D., Vopper, G., Boned, A., Goridis, C., and Wille, W. (1989) *EMBO J.* **8**, 385–392
35. Gresham, H. D., Goodwin, J. L., Allen, P. M., Anderson, D. C., and Brown, E. J. (1989) *J. Cell Biol.* **108**, 1935–1943
36. Yuan, Q., Jiang, W.-M., Krissansen, G. W., and Watson, J. D. (1990) *Int. Immunol.* **2**, 1097–1108

Ciba Foundation Symposium 189

CELL ADHESION AND HUMAN DISEASE

1995

JOHN WILEY & SONS

Chichester · New York · Brisbane · Toronto · Singapore

Mechanisms of VCAM-1 and fibronectin binding to integrin $\alpha_4\beta_1$: implications for integrin function and rational drug design

Martin J. Humphries, Joe Sheridan, A. Paul Mould and Peter Newham

School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK

Abstract. Integrin $\alpha_4\beta_1$ can mediate both cell–cell and cell–extracellular matrix adhesion by binding to either fibronectin or vascular cell adhesion molecule 1 (VCAM-1). Both interactions are important for extravasation of leukocytes from the blood implying that rationally designed inhibitors of $\alpha_4\beta_1$ function may be useful for treating various inflammatory conditions. The mechanisms of ligand binding by $\alpha_4\beta_1$ are complicated by the fact that alternative splicing can generate different isoforms of the receptor-binding domains in both fibronectin and VCAM-1. Therefore, in addition to developing $\alpha_4\beta_1$ antagonists, we have also been interested in identifying isoform-specific functions. Recombinant ligand variants have been tested in adhesion and direct receptor-binding assays and each molecule was found to have a different inherent affinity for $\alpha_4\beta_1$ that endows them with different adhesive activities. This suggests that alternative splicing may regulate $\alpha_4\beta_1$-dependent motility *in vivo*. The initial strategy that we have adopted to develop $\alpha_4\beta_1$ inhibitors has been to identify key amino acid residues and peptide sequences participating in the receptor–ligand binding event and to use this information to generate synthetic mimetics. Three active sites have been identified in fibronectin by testing truncated proteins, expressing recombinant fragments and screening synthetic peptides. Two of these sites employ versions of a novel integrin-binding motif, LDVP/IDAP. A key active site in VCAM-1 has been identified by similar approaches as the related sequence IDSP. Since IDSP-like sequences are probably used by other integrin-binding immunoglobulins, derivatives of these peptides may turn out to be the forerunners of a new generation of therapeutic agents with multiple applications.

1995 Cell adhesion and human disease. Wiley, Chichester (Ciba Foundation Symposium 189) p 177–194

Adhesion is a fundamental prerequisite for many normal cell functions. This need is most commonly exemplified by the dependence of growth on anchorage, but adhesion also generates the traction required for cell movement

and cell positioning, and directly influences intracellular signalling systems and gene expression. In addition to normal functions, adhesion also contributes to the pathogenesis of many of the most common human diseases. In these cases, if cell-adhesive interactions could be regulated, then disease progression might be arrested. Prime molecular targets for the generation of adhesion regulators are adhesion receptors, of which integrins are the principal class. Integrins are a family of α, β heterodimeric glycoproteins that currently contains 21 different members in vertebrates (Hynes 1992). Each dimer possesses a unique ligand specificity, suggesting that different receptors exhibit different functions *in vivo* and implying that specific integrin antagonists could have many applications (Humphries 1990).

The first step in the rational design of adhesion regulators requires information about the structure of adhesion molecules. Ideally, this would be a three-dimensional description of the receptor–ligand binding complex, but, alternatively, active site domains of either the receptor or ligand could be studied in isolation. In recent years, the rational design of integrin antagonists has been aided immensely by studies of the molecular basis of ligand binding. A key concept that has emerged from this work is that the majority of integrin ligands use very short protein sequences as receptor recognition motifs (Humphries 1990, Yamada 1991). Analogues of these sequences are essentially lead compounds for drug design (Humphries et al 1994). Initial studies, using fibronectin as a model, mapped adhesive domains using proteolytic fragments to the point at which it was possible to reproduce activity in the form of synthetic peptides. This important strategic transition permitted extremely rapid elucidation of a key minimal active sequence as the tripeptide RGD (Pierschbacher & Ruoslahti 1984). The discovery that peptides containing such a short sequence could possess adhesive activity has since been substantiated by extensive antibody and mutagenesis studies of fibronectin, and by the fact that other adhesion molecules, including fibrinogen, von Willebrand factor, vitronectin and osteopontin have subsequently been shown to employ the same RGD motif, albeit with different receptor specificity profiles (Humphries 1990).

RGD-dependent binding of fibrinogen to the platelet integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa), an interaction that plays a key role in thrombosis, is the first integrin–ligand interaction to be targeted by pharmaceutical companies for drug development. This work has now led to the development of RGD mimetics which exhibit potency, specificity and a capability for oral delivery (Humphries et al 1994). Current indications from clinical trials with these antagonists in unstable angina, coronary angioplasty and myocardial infarction are encouraging. In addition to being used in soluble form, the RGD motif has also been incorporated into formulations with hydrophobic peptide linkers or glycosaminoglycans to enhance cell adhesion. Initial clinical trial data from the treatment of ulcers and tissue grafts have shown positive effects on the acceleration of wound closure, improvement of cellular organization and reduction of scarring.

Subsequent to the discovery of the RGD recognition sequence, the molecular basis of other integrin–ligand interactions has been partially solved. In three cases activity can again be described by synthetic peptides: first, proteolytic dissection of fibrinogen has led to the identification of a peptide from the C-terminus of the γ-chain with a minimal sequence QAGDV which binds to $\alpha_{IIb}\beta_3$ (Kloczewiak et al 1984); second, the major integrin $\alpha_4\beta_1$-binding site in fibronectin has been localized to the tripeptide LDV (Komoriya et al 1991); and third, evidence has been presented to implicate the sequence DGEA as an active site in collagen type I for binding to the integrin $\alpha_2\beta_1$ (also known as GPIa/IIa) (Staatz et al 1991). There appears to be a functional analogy between some of these motifs since QAGDV and RGD peptides bind to the same or mutually exclusive binding sites on $\alpha_{IIb}\beta_3$ (Santoro & Lawing 1987) and LDV and RGD have the same relationship for binding $\alpha_4\beta_1$ (Mould et al 1991). This suggests that the concepts learned from studies of RGD may be applicable to other integrin-binding motifs.

The integrin $\alpha_4\beta_1$ and its ligands

In recent years, work in this laboratory has focused on the leukocyte integrin $\alpha_4\beta_1$ (also known as VLA-4) and its two ligands, the extracellular matrix glycoprotein fibronectin (Wayner et al 1989, Guan & Hynes 1990, Mould et al 1990) and the endothelial cell-surface protein VCAM-1 (Elices et al 1990). Since anti-$\alpha_4\beta_1$ monoclonal antibodies have been shown to inhibit trafficking of leukocytes in a number of acute and chronic inflammatory conditions *in vivo*, it appears that these interactions contribute to leukocyte extravasation and are therefore important therapeutic targets (Yednock et al 1992, Weg et al 1993). Consequently, we have been interested in determining the molecular basis of $\alpha_4\beta_1$–ligand binding with a view to using this information to develop specific antagonists.

In addition to the role of $\alpha_4\beta_1$ in disease processes, results from several studies have also identified $\alpha_4\beta_1$–ligand interactions as a useful model system for studying the regulation of integrin-mediated migration: first, $\alpha_4\beta_1$ is generally expressed by highly motile cell types such as leukocytes and neural crest derivatives, and is employed during migration of these cells *in vitro* (Hemler et al 1990); second, expression of $\alpha_4\beta_1$ by tumour cells correlates with invasiveness and metastatic potential (Albelda et al 1990); third, expression of a chimeric integrin containing the α_2 extracellular domain and the α_4 cytoplasmic domain enhances the ability of cells to migrate on collagen substrates compared to cells transfected with chimaeras containing other cytoplasmic domains (Chan et al 1992). It is therefore conceivable that an understanding of the role of $\alpha_4\beta_1$ in cell migration will yield insights that are applicable to other adhesion systems.

$\alpha_4\beta_1$ binding to fibronectin occurs in a complex manner via the HepII/IIICS region. HepII is the major proteoglycan-binding domain of fibronectin, while

IIICS is one of three sites subject to alternative splicing within the molecule. Three distinct $\alpha_4\beta_1$-binding sites have been identified within the HepII/IIICS region (Humphries et al 1986, 1987, Mould & Humphries 1991). Two sites (represented by peptides CS-1 and CS-5) are present in independently spliced segments of the IIICS (Humphries et al 1986, 1987), while the other site (represented by the peptide H-1) is found in the HepII region and is therefore expressed in all fibronectin isoforms (Mould & Humphries 1991). As discussed above, the CS-1 peptide contains the tripeptide LDV as its minimal active site (Komoriya et al 1991). H-1 contains a related motif, IDA, while CS-5 incorporates a variant of RGD, REDV (Humphries et al 1986, Mould & Humphries 1991). On the basis of previous studies of the relative activities of the three active site peptides, CS-1 has approximately 20-fold greater activity than CS-5 and the activity of H-1 is similar to that of CS-5 (Mould & Humphries 1991, Mould et al 1991). VCAM-1 is a member of the immunoglobulin superfamily, consisting of six or seven immunoglobulin repeats. The sites in VCAM-1 recognized by $\alpha_4\beta_1$ lie within immunoglobulin domains I and IV (Vonderheide & Springer 1992). In the six-domain form of VCAM-1, domain IV is removed by alternative splicing.

Functional aspects of $\alpha_4\beta_1$–ligand binding

The location of integrin binding sites in alternatively spliced segments of both $\alpha_4\beta_1$ ligands suggests that splicing may regulate function. To test this possibility, we adopted the approach of generating recombinant versions of the HepII/IIICS splice variants. Four variants have been studied which contain the four possible combinations of the three active sites—CS-1, CS-5 and H-1. The H-120 variant contains all three known sites, H-89 contains CS-1 and H-1, H-95 contains CS-5 and H-1, and H-0 contains H-1 alone (Fig. 1). When tested in cell attachment and spreading assays, the relative activities of the different variants mirrored the relative activities of CS-1, CS-5 and H-1 peptides—i.e. if present, the CS-1 sequence dominated the activity of the HepII/IIICS region (Mould et al 1994). The difference in activity between H-95 and H-0 implies that the CS-5 site is active in the H-95 variant.

Interestingly, inhibition experiments with anti-integrin antibodies suggested that whereas adhesion to VCAM-1, H-120 and H-89 was almost exclusively mediated by $\alpha_4\beta_1$, some of the cell attachment and migration mediated by H-95 and H-0 was not due to integrin recognition. Instead, attachment was partially inhibited by heparin, suggesting that proteoglycans were also mediating cell adhesion and that proteoglycans and $\alpha_4\beta_1$ may cooperate in promoting attachment to the HepII domain. In contrast, we observed that heparin did not significantly inhibit attachment to H-120 and H-89, suggesting that proteoglycan binding does not influence recognition of this sequence by $\alpha_4\beta_1$.

FIG. 1. Schematic representation of the four different recombinant variants of the HepII/IIICS region referred to in the text (H variants). The top part of the figure shows the modular structure of the fibronectin subunit: closed boxes, type I repeats; cross-hatched boxes, type II repeats; hatched boxes, type III repeats; open boxes, regions subject to alternative splicing. The methods used for polymerase chain reaction cloning and expression of H variants are given in Makarem et al (1994). The numbers assigned to H variants refer to the number of IIICS residues in the protein. The locations of the three recognition sequences for $\alpha_4\beta_1$, H-1, CS-1 and CS-5 are indicated.

Measurements of receptor binding affinities for the different recombinant proteins in a solid-phase binding assay in the presence of 1 mM $MnCl_2$ showed that VCAM-1 bound to $\alpha_4\beta_1$ with approximately fourfold higher affinity than H-120 and H-89 (which were both of similar affinity). H-120 exhibited approximately 10-fold higher binding affinity than H-95, which in turn exhibited approximately twofold higher affinity than H-0 (Mould et al 1994). These results match the observed differences between the ligands in adhesion assays. An important conclusion from these studies is that the effects of fibronectin and VCAM-1 on cell adhesion appear to depend only on the affinity of their interactions with $\alpha_4\beta_1$ and not on a ligand-specific sequence.

The significance of differences in affinity could be related to receptor reorganization with high-affinity ligand binding eliciting clustering of larger numbers of integrins compared to low-affinity ligand binding, and therefore to more stable interactions of integrins with the cytoskeleton. Alternatively, high-affinity ligand binding may transduce different signals to the cell interior compared to low-affinity binding, for example through effects on integrin conformation, clustering, or association of signalling molecules. *In vivo*, the high affinity binding of VCAM-1 to $\alpha_4\beta_1$ may favour the arrest of leukocytes on the surface of endothelial cells at sites of inflammation.

A novel finding from measurements of the kinetics of $\alpha_4\beta_1$–ligand binding was that changes in ligand affinity were due principally to changes in the rate of ligand–receptor association (k_1) rather than ligand–receptor dissociation (k_{-1}). The activation energies for these reactions are likely to derive mainly from conformational changes in both ligand and receptor; the identification of conformation-specific monoclonal antibodies has indicated that such changes do occur (Frelinger et al 1990, Zamarron et al 1991). The results from $\alpha_4\beta_1$–ligand binding assays suggest that receptor–ligand engagement requires changes in ligand conformation (these changes may only be small for sequences in VCAM-1, but very large for low-affinity sequences, such as in the HepII region of fibronectin). In contrast, receptor–ligand dissociation appears to require changes in receptor conformation.

Recombinant fibronectin and VCAM-1 ligands were also examined for their ability to promote melanoma cell migration. Migration on H-120, H-89 and VCAM-1 showed a biphasic response as a function of the coating concentration of ligand, with recombinant soluble VCAM-1 being two- to threefold more potent in the first phase. Higher concentrations of all ligands supported reduced cell migration. Our interpretation is that a threshold level of cell adhesiveness is needed for cell migration to start, at intermediate adhesiveness cell motility increases to a maximum and at high adhesiveness migration declines as a result of the inability of the cells to break contact with the substrate. Because adhesiveness is likely to be directly proportional to the affinity of receptor–ligand binding, the results can again be explained by the differences in ligand affinities. For H-95, which promotes weak migration that is not reduced at high coating concentration, it appears that its adhesive strength is too weak for cell migration to reach a maximum and, for H-0, the threshold level at which migration can begin is not attained.

We previously hypothesized that adhesion to lower-affinity sites, such as CS-5, may allow more rapid cell migration, whereas adhesion to the high-affinity CS-1 site may restrict cell migration. The results presented here suggest the opposite and point to a key role for CS-1 in migration. However, the concentration of fibronectin in the extracellular matrix is likely to be of crucial importance *in vivo*. If high concentrations of fibronectin containing the CS-1 site are present, the attachment strength may exceed that required for optimal cell migration. Enforced inclusion of different spliced segments of the IIICS region through transgenic approaches may provide additional insights into the effects of the different $\alpha_4\beta_1$ recognition sites on migration *in vivo*.

VCAM-1 active sites

As described above, VCAM-1-mediated leukocyte extravasation is an important therapeutic target for the development of anti-inflammatory agents. Recently, we have shown that fibronectin and VCAM-1 act as competitive inhibitors of

each other's binding to $\alpha_4\beta_1$ (Makarem et al 1994). This suggests that $\alpha_4\beta_1$ may interact with fibronectin and VCAM-1 by a similar mechanism. The tripeptide sequence LDV is the key active sequence within CS-1 (Komoriya et al 1991) and, on inspection, VCAM-1 domains I and IV contain a related sequence, I(39)DSP. A computer-generated model of VCAM-1 domain I, based on immunoglobulin domain crystal structures, places this sequence in the exposed C–D loop region that is potentially accessible to $\alpha_4\beta_1$ (Fig. 2). Molecular dynamics simulations predict that the IDSP loop is flexible and projected away from the side of the domain.

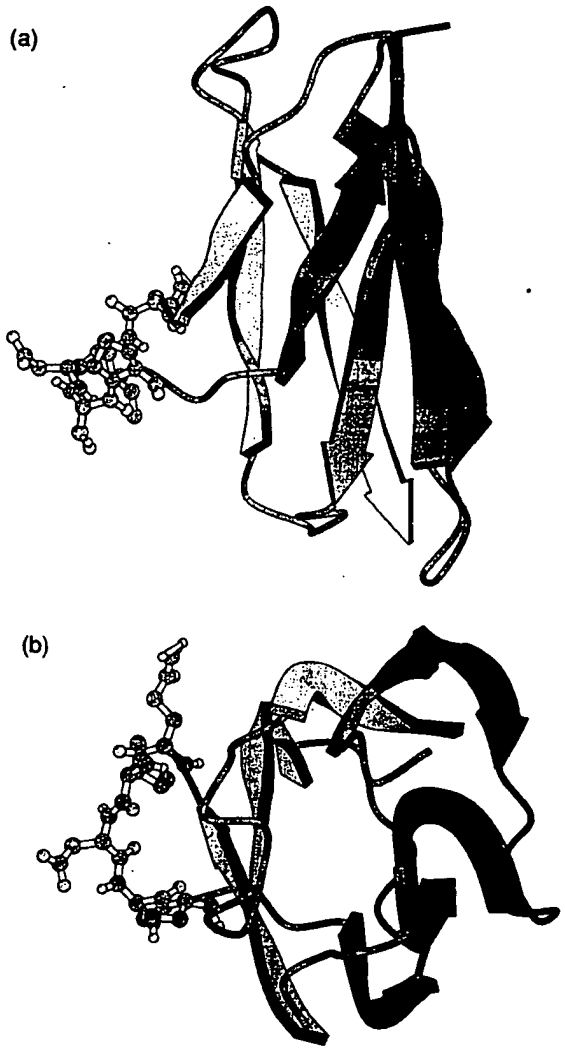


FIG. 2. Model of VCAM-1 domain I. The model shown is an averaged structure following molecular dynamics simulations and is based on the Ig crystal structure of 1REIa (for further details see Clements et al 1994). The diagram was produced with the aid of the display program MOLSCRIPT (Kraulis 1991). The β-strands that collectively form the ABDE sheet are shown as dark grey arrows and those that form the CFG sheet are shown as light grey arrows. The I(39)DSP sequence is shown in ball-and-stick form. In (a) the domain is viewed from the side; in (b) it is viewed from the top.

A number of mutations within the IDSP-containing loop and within other sequences predicted to be surface-accessible, were screened for their effects on adhesive activity. Using a simplified VCAM-1 construct lacking domains IV–VI, mutation of the I(39)DSP sequence essentially abrogated $\alpha_4\beta_1$–VCAM-1 binding (Clements et al 1994). The profile of monoclonal antibody binding to mutated VCAM-1 molecules was not affected by the mutation, indicating that the effects on adhesion were a direct consequence of perturbing integrin recognition. To examine the contribution of the IDSP sequence in domain IV of VCAM-1, we made use of the fact that $\alpha_4\beta_1$ adhesion to VCAM-1 exhibits temperature sensitivity; binding to domain I will take place at either 37 °C or 4 °C, while binding to domain IV only takes place at 37 °C (Needham et al 1994). Mutations in full length VCAM-1 within the IDSP region of domain I only partially reduced adhesion at 37 °C (Clements et al 1994). When adhesion was tested at 4 °C, almost all binding was abolished, indicating that the residual activity observed at 37 °C could be attributed to $\alpha_4\beta_1$ interaction with domain IV. Mutations of the I(327)DSP sequence of domain IV had no significant effect at either assay temperature, indicating that if I(327)DSP is active, $\alpha_4\beta_1$ preferentially binds domain I over domain IV. Simultaneous mutation of both IDSP sequences in domains I and IV produced a molecule that was unable to support adhesion at either temperature. Taken together, these data suggest that $\alpha_4\beta_1$ binds domain I and domain IV via a common mechanism mediated by the IDSP motif. However, if binding to one domain is compromised, binding can still take place via the other domain.

In agreement with the data from mutagenesis experiments, a peptide spanning the IDSP region (TQIDSPLN) was shown to inhibit spreading of melanoma cells on recombinant soluble VCAM-1 in a dose-dependent manner (Clements et al 1994). The peptide possessed similar activity to the fibronectin CS-1 peptide, while a control peptide with scrambled sequence (QNLSPITD) had no significant inhibitory effect. A peptide containing IDSP can therefore specifically and effectively perturb $\alpha_4\beta_1$–VCAM-1 interactions, thereby confirming the results obtained from VCAM-1 mutagenesis. Importantly, this result suggests that the mechanism of integrin binding by immunoglobulin ligands is related to that employed by most extracellular matrix ligands.

Although much structure–activity work remains to be performed, the IDSP motif is the third LDV-like sequence to be identified as a functional integrin-binding site (after the prototypic LDV(P) site in CS-1 and the IDA(P) sequence in the fibronectin HepII peptide H-1). A series of other reports suggest that there may be many more examples of the use of similar sequences and that this is in fact a second common integrin-binding motif, functionally related to RGD. One such sequence is the QAGDV peptide from the C-terminus of the γ-chain of fibrinogen (Kloczewiak et al 1984). This peptide bears some homology to LDV (Table 1) and, like LDV (Humphries et al 1987), is a competitive inhibitor of RGD function (Santoro & Lawing 1987). It is most striking, however, that

TABLE 1 Sequence homology in domain I C–D loop region of integrin-binding cell adhesion molecules and comparison with functionally active sequences in fibronectin and fibrinogen.

Protein	*Species*	*Sequence*
FN CS-1	Hu/Ra/Bo	PEI **LDVP** STV
FN CS-1	Ch	PDM **LDVP** SVD
FN CS-1	Xe	PEI **LDVP** TDE
FN H-1	Hu/Ra/Bo/Ch	STA **IDAP** SNL
FN H-1	Xe	TTA **VDSP** SNL
FG γ-chain	Hu	KQA **GDV.**
VCAM-1 DI	Hu/Ra	RTQ **IDSP** LNG
VCAM-1 DI	Mu	RTQ **IDSP** LNA
VCAM-1 DIV	Hu	RTQ **IDSP** LSG
VCAM-1 DIV	Mu/Ra	RTQ **TDSP** LNG
MAdCAM-1 DI	Hu	WRG **LDTS** LGS
ICAM-1 DI	Hu	LLG **IETP** LPK
ICAM-1 DI	Mu	SLG **LETQ** WLK
ICAM-1 DI	Ra	GLG **LETN** WMK
ICAM-2 DI	Hu	VGG **LETS** LNK
ICAM-2 DI	Mu	MGG **LETP** TNK
ICAM-3 DI	Hu	KIA **LETS** LSK

'LDVP' homology is in bold. FN, fibronectin; FG, fibrinogen; Hu, human; Ra, rat; Bo, bovine; Ch, chicken; Xe, *Xenopus*; Mu, mouse.

all other integrin-binding immunoglobulin ligands contain either an aspartate or a glutamate residue in their membrane-distal domains in a position analogous to the IDSP of VCAM-1 (Table 1). There is now considerable evidence that each of these residues contributes to function: mutation of (i) E34 within ICAM-1 domain I (Staunton et al 1990) and (ii) E37 within ICAM-3 (D. Simmons, personal communication) substantially inhibits $\alpha_L\beta_2$ (LFA-1) binding; (iii) mutation of D41 within MAdCAM-1 inhibits $\alpha_4\beta_7$ binding (M. Briskin, personal communication); and (iv) a peptide spanning the L(39) ETP site of of ICAM-2 is an effective inhibitor of $\alpha_L\beta_2$ binding and is capable of a direct interaction with $\alpha_L\beta_2$ (Li et al 1993). Thus, a consensus integrin-binding motif on immunoglobulin cell adhesion molecules becomes apparent: aliphatic–aspartate or glutamate–serine or threonine–proline or hydrophilic. The use of a glutamate rather than an aspartate residue by ICAMs contrasts with the absolute dependence of RGD sequences on aspartate, but might reflect a slightly deeper active site pocket in β_2 integrins. In the future, it will be of

pressing importance to test the ability of LDV-like ('LDV') peptides to perturb each of these integrin–ligand interactions using sensitive assays for adhesion, since this would have implications for the design of novel classes of anti-adhesive agent.

One question prompted by the common use of RGD- and LDV-like sequences is the extent to which each integrin–ligand interaction is dependent on a short acidic peptide motif. In the case of fibrillar collagen molecules, which bind to $\alpha_1\beta_1$ and $\alpha_2\beta_1$, linear sequences may not be involved. Using the adhesion of human chondrosarcoma cells to type II collagen as a model system, we have found that native and heat-denatured collagen molecules support adhesion by different mechanisms. Recognition of native type II collagen by $\alpha_2\beta_1$ requires a triple helical conformation and is not inhibited by linear CNBr fragments or by the DGEA peptide described by Staatz et al (1991). Recognition of denatured collagen, on the other hand, is mediated by a fibronectin–$\alpha_5\beta_1$ bridge, not by $\alpha_2\beta_1$, and is inhibited by CNBr fragments. The inability to reproduce the integrin-binding activity of type II collagen in the form of a linear peptide implies that collagens employ a different type of active site in their receptors to those ligands that use RGD- or LDV-like sequences. This appears to be the case, since in recent work (D. S. Tuckwell and M. J. Humphries, unpublished results), we have shown that the recombinant I-domain of α_2 interacts specifically with fibrillar collagen in a cation-dependent manner.

Future prospects: integrin-binding peptide potency and specificity

The fact that both RGD and LDV are used as common recognition signals provides a partial explanation for the ligand-binding promiscuity of integrins. It also explains why an integrin ligand can competitively inhibit the binding of other ligands to the same integrin, e.g. fibrinogen, fibronectin and von Willebrand factor for $\alpha_{IIb}\beta_3$, and fibronectin and VCAM-1 for $\alpha_4\beta_1$. Some integrins, however, exhibit exquisite ligand-binding specificity, implying that either the integrins, the ligands, or both have structural features that generate this specificity. Little is known about integrin structure, but as far as the ligands are concerned, two possibilities are that the peptides themselves are held in different conformations in different ligands, or that other regions of the ligands contribute to receptor binding and endow specificity. Although there is some evidence supporting the latter view (Obara et al 1988, Bowditch et al 1994), it is now clear that simple alterations in the structure of synthetic RGD peptides can produce molecules that are selective inhibitors. In turn, this implies that normal flanking/spatially adjacent sequences in the ligand may serve to constrain RGD conformation. As yet, in the absence of crystal structure data, there are few indications as to how this is achieved. However, one reproducible finding from RGD structure–function studies has been the generation of greater potency in RGD peptides containing highly hydrophobic amino acids in the position following the aspartate (e.g. L, Y, F and W). This suggests that hydrophobic

bonding may contribute to the interaction of RGD sequences with their integrin active sites. How this requirement relates to the hydrophobic residues in LDV motifs remains to be investigated. What is apparent so far from LDV structure–function studies is that the motif has greater tolerance of amino acid substitutions in the residues flanking the key aspartate. This offers hope that peptide engineering might produce selective inhibitors for cell adhesion molecule-binding integrins. As expected from work with RGD, linear LDV peptides are predicted to be extremely flexible (Fig. 3) and studies are now underway to introduce conformational restrictions into these peptides, initially by cyclization.

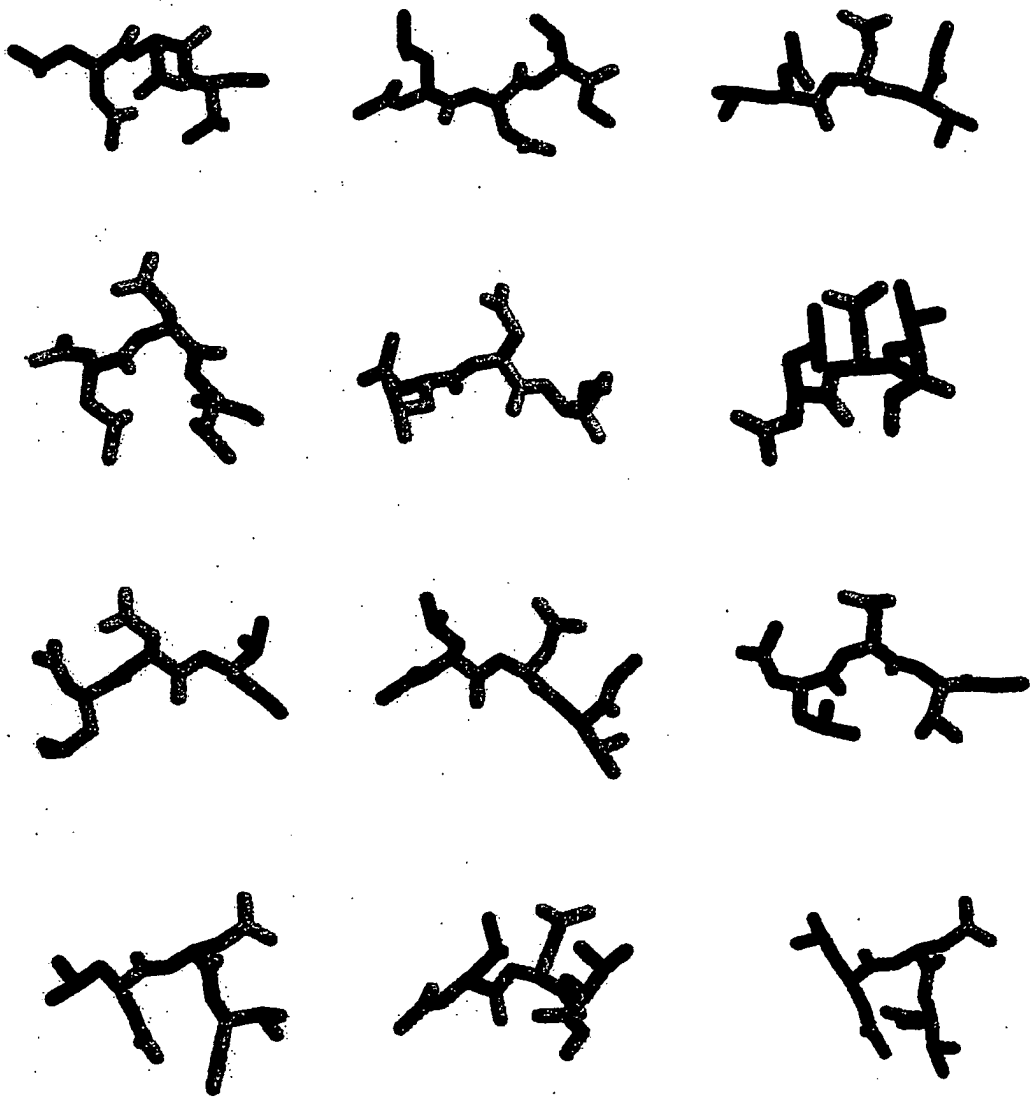


FIG. 3. Accessible low-energy conformers of Ac-LDV-N-methyl amide after molecular dynamics-simulated annealing at an exhaustively slow cooling rate (12.5 K/ps; J. Sheridan & M. J. Humphries, unpublished results). The high variability in low-energy structures demonstrates the flexibility of the linear peptide.

Tertiary structure determinations of RGD sites in fibronectin (Fig. 4), tenascin, kistrin, echistatin and recently in the foot-and-mouth disease VP1 coat protein have in all cases localized them to flexible surface loops (Adler et al 1991, Chen et al 1991, Leahy et al 1992, Main et al 1992, Logan et al 1993). Interestingly, although we cannot comment on flexibility, our prediction is that LDV active sites in integrin-binding immunoglobulins are also localized to loops. This location may be functionally important in endowing a relatively high on-rate for ligand binding or may reflect the fact that the majority of the loop is not anchored to the rest of the molecule, but it also suggests that there is likely to be a high degree of induced fit in the ligand subsequent to binding. Elucidation of the conformational changes that take place in both ligands and receptors during binding and dissociation, and how this varies between ligand isoforms, remains a fundamentally important question. In addition, further investigation of the molecular basis and functional consequences of ligand binding by the integrin $\alpha_4\beta_1$ promises to answer a number of other key questions.

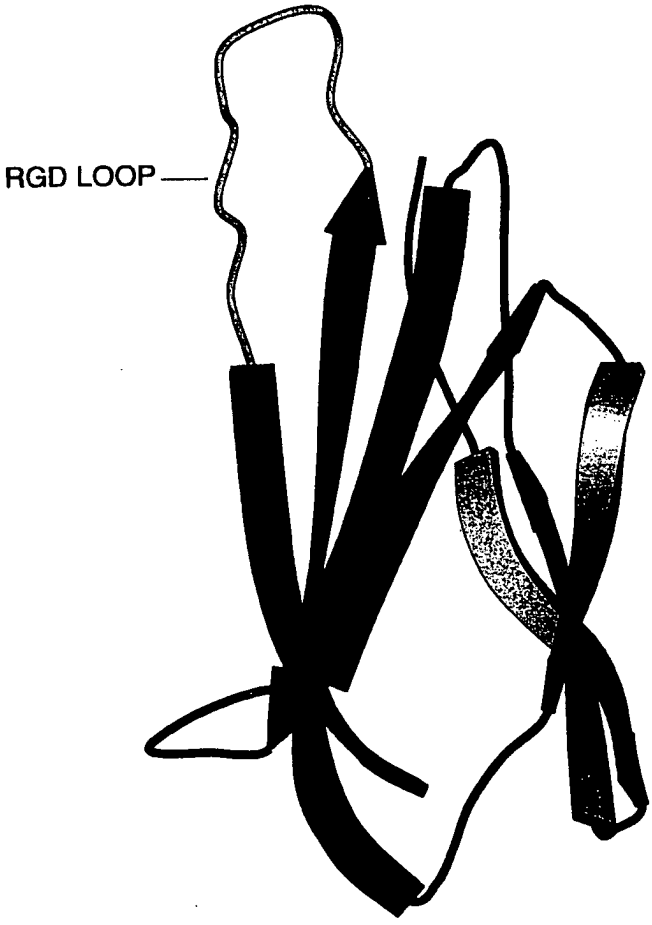


FIG. 4. Model of fibronectin type III repeat 10. The model is derived from atomic coordinates based on results from nuclear magnetic resonance studies of the recombinant molecule (Main et al 1992). The location of the RGD site is indicated.

For example, how large is the family of LDV-containing proteins? What are the essential structural features of an active 'LDV' sequence and can these be exploited to permit development of a novel family of specific integrin antagonists? What is the functional role of the short aspartate- or glutamate-containing peptides employed by integrin ligands and how is this related to the nature of integrin active sites?

References

Adler M, Lazarus RA, Dennis MS, Wagner G 1991 Solution structure of kistrin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist. Science 253:445–448

Albelda SM, Mette SA, Elder DE et al 1990 Integrin distribution in malignant melanoma: association of the $\beta 3$ subunit with tumor progression. Cancer Res 50:6757–6764

Bowditch RD, Hariharan M, Tominna EF et al 1994 Identification of a novel integrin binding site in fibronectin. Differential utilization by $\beta 3$ integrins. J Biol Chem 269:10856–10863

Chan BMC, Kassner PD, Schiro JA, Byers HR, Kupper TS, Hemler ME 1992 Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell 68:1051–1060

Chen Y, Pitzenberger SM, Garsky VM, Lumma PK, Sanyal G, Baum J 1991 Proton NMR assignments and secondary structure of the snake venom protein echistatin. Biochemistry 30:11625–11636

Clements JM, Newham P, Shepherd M et al 1994 Identification of a key integrin-binding sequence in VCAM-1 homologous to the LDV active site in fibronectin. J Cell Sci 107:2127–2135

Elices MJ, Osborn L, Takada Y et al 1990 VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 60:577–584

Frelinger AL, Cohen I, Plow EF et al 1990 Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. J Biol Chem 265:6346–6352

Guan J-L, Hynes RO 1990 Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell 60:53–61

Hemler ME, Elices MJ, Parker C, Takada Y 1990 Structure of the integrin VLA-4 and its cell–cell and cell–matrix adhesion functions. Immunol Rev 114:45–65

Humphries MJ 1990 The molecular basis and specificity of integrin–ligand interactions. J Cell Sci 97:585–592

Humphries MJ, Akiyama SK, Komoriya A, Olden K, Yamada KM 1986 Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. J Cell Biol 103:2637–2647

Humphries MJ, Komoriya A, Akiyama SK, Olden K, Yamada KM 1987 Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. J Biol Chem 262:6886–6892

Humphries MJ, Doyle PM, Harris CJ 1994 Integrin antagonists as modulators of adhesion. Expert Opin Ther Pat 4:227–235

Hynes RO 1992 Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25

Kloczewiak M, Timmons S, Lukas TJ, Hawiger J 1984 Platelet receptor recognition site on human fibrinogen. Synthesis and structure–function relationship of peptides corresponding to the carboxy-terminal segment of the γ-chain. Biochemistry 23:1767–1774

Komoriya A, Green LJ, Mervic M, Yamada SS, Yamada KM, Humphries MJ 1991 The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. J Biol Chem 266:15075–15079

Kraulis PJ 1991 MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 24:946–950

Leahy DJ, Hendrickson WA, Aukhil I, Erickson HP 1992 Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. Science 258:987–991

Li R, Nortamo P, Valmu L et al 1993 A peptide from ICAM-2 binds to the leukocyte integrin CD11a/CD18 and inhibits endothelial cell adhesion. J Biol Chem 268: 17513–17518

Logan D, Abu-Ghazaleh R, Blakemore W et al 1993 Structure of a major immunogenic site on foot-and-mouth disease virus. Nature 362:566–568

Main AL, Harvey TS, Baron M, Boyd J, Campbell ID 1992 The three-dimensional structure of the tenth type III module of fibronectin: an insight into RGD-mediated interactions. Cell 71:671–678

Makarem R, Newham O, Askari JA et al 1994 Competitive binding of vascular cell adhesion molecule-1 and the HepII/IIICS domain of fibronectin to the integrin $\alpha4\beta1$ J Biol Chem 269:4005–4011

Mould AP, Humphries MJ 1991 Identification of a novel recognition sequence for the integrin $\alpha4\beta1$ in the carboxy-terminal heparin-binding domain of fibronectin. EMBO (Eur Mol Biol Organ) J 10:4089–4095

Mould AP, Wheldon LA, Komoriya A, Wayner EA, Yamada KM, Humphries MJ 1990 Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha4\beta1$. J Biol Chem 265:4020–4024

Mould AP, Komoriya A, Yamada KM, Humphries MJ 1991 The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin $\alpha4\beta1$. Inhibition of $\alpha4\beta1$ function by RGD peptide homologues. J Biol Chem 266: 3579–3585

Mould AP, Askari JA, Craig SE, Garratt AN, Clements J, Humphries MJ 1994 Cell adhesion and migration mediated by the integrin $\alpha4\beta1$: effect of fibronectin alternative splicing and the role of ligand affinity. J Biol Chem, in press

Needham LA, Van Dijk S, Pigott R et al 1994 Activation dependent and independent binding sites on vascular cell adhesion molecule-1. Cell Adhes Commun 2:87–99

Obara M, Kang MS, Yamada KM 1988 Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable synergistic sites mediate adhesive function. Cell 53:649–657

Pierschbacher MD, Ruoslahti E 1984 Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309:30–33

Santoro SA, Lawing WJ 1987 Competition for related but non identical binding sites on the glycoprotein IIb-IIIa complex by peptides derived from platelet adhesive proteins. Cell 48:867–873

Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santora SA 1991 Identification of a tetrapeptide recognition sequence for the $\alpha2\beta1$ integrin in collagen. J Biol Chem 266:7363–7367

Staunton DE, Dustin ML, Erickson HP, Springer TA 1990 The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell 61:243–254

Vonderheide RH, Springer TA 1992 Lymphocyte adhesion through very late antigen 4: evidence for a novel binding site in the alternatively spliced domain of vascular cell adhesion molecule 1 and an additional $\alpha 4$ integrin counter-receptor on stimulated endothelium. J Exp Med 175:1433–1442

Wayner EA, Garcia-Pardo A, Humphries MJ, McDonald JA, Carter WG 1989 Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain CS-1 in plasma fibronectin. J Cell Biol 109:1321–1330

Weg VB, Williams TJ, Lobb RR, Nourshargh S 1993 A monoclonal antibody recognizing very late activation antigen-4 inhibits eosinophil accumulation *in vivo*. J Exp Med 177:561–566

Yamada KM 1991 Adhesive recognition sequences. J Biol Chem 266:12809–12812

Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N 1992 Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin. Nature 356:63–66

Zamarron C, Ginsberg MH, Plow EF 1991 A receptor-induced binding site in fibrinogen elicited by its interaction with platelet membrane glycoprotein IIb-IIIa. J Biol Chem 266:16193–16199

DISCUSSION

Hynes: You don't see any inhibition of adhesion to collagen by the peptide DGEA, so how do you explain Santoro's results (Staatz et al 1991), which look pretty convincing? Do you understand why yours are different?

Humphries: No.

Sonnenberg: I'm not convinced that the peptide used by Santoro is the only relevant peptide sequence.

Hynes: It may not be the *only* one, but Santoro's data that it was an $\alpha_2\beta_1$ inhibitor weren't bad.

Humphries: The concentrations of peptide that they used were very high. In order to see activity with a truncated peptide, I believe they needed concentrations of 5–6 mM.

Hynes: Do you see the I-domain of $\alpha_2\beta_1$ binding to laminin as well? This is also a laminin receptor in some situations.

Humphries: No, we didn't see any binding with the α_2 I-domain.

Sonnenberg: Can you block $\alpha_1\beta_1$ binding to collagen with the I-domain of α_2?

Humphries: We've not tried that.

Rothlein: Did you look for the integrin-binding sequence in domain III of ICAM-1, which binds to $\alpha_M\beta_2$ (Mac-1)?

Humphries: Yes; there doesn't appear to be a homologous sequence. I don't know what that says about ICAM-1–$\alpha_M\beta_2$ binding.

Rothlein: It seems pretty clear that when you have an I-domain, antibodies that block cell adhesion bind to the I-domain; antibodies that don't bind to the I-domain are not effective blockers of cell adhesion, yet most anti-β-subunit

antibodies block effectively. What's the relationship between the α and the β subunits?

Humphries: That's a central question. We desperately need to understand how the ligand-binding pocket is formed. The information could also impact upon activation, drug development and all kinds of phenomena. I should add that there are examples of antibodies that don't bind to the I-domain blocking function. Danny Tuckwell has found that HAS-6, one of Fiona Watt's anti-α_2 antibodies, is a weak blocker of adhesion and doesn't map to the I-domain (Tenchini et al 1993).

Of course, we should remember that the I-domain isn't found in every integrin and therefore can't explain all ligand-binding events.

I should also add that Klaus Kuhn's lab have reported a quite interesting hypothesis about how type IV collagen is recognized by $\alpha_1\beta_1$ (Eble et al 1993). They're lucky that they can actually fragment type IV collagen and retain a triple helical structure that is held together by disulphide bonds. By chemical modification of amino acids, they've shown that there is an arginine residue and an aspartate residue that are crucial for that binding. However, these two residues are not on the same chain in the trimer, but are actually found on adjacent chains. They think that there may well actually be an RGD type of recognition of the type IV collagen mediated by a basic and an acidic group on different chains.

Sonnenberg: But your isolated I-domain doesn't bind to collagen IV?

Humphries: It binds, but weakly. It also binds weakly to type VI, which is very interesting.

Sonnenberg: How do you explain that? Are there two binding sites?

Humphries: I think there's more than one binding site and that, depending on the ligand, each is used to a different extent.

Elices: What is the relationship between the solid-phase assays you do (in which you use the purified receptor) and the receptor that is expressed on cells?

Humphries: Fundamentally they are the same. The binding we see in the solid-phase assays is specific and occurs under conditions that support adhesion to authentic ligands. In cell-based assays the environment of the integrin might be modulated to produce receptors with different activities. This is where cell adhesion experiments can actually give different results. For example, differences in receptor presentation and in intracellular activation or clustering of the integrin can give you apparent differences in cation sensitivity or sensitivity of the adhesion to inhibitors.

Shaltiel: Can you tell us more about the effect of the cations on the efficacy of the different peptides?

Humphries: It's dependent on the integrin. In a way it's quite reassuring to see a different pattern for the α_2 I-domain and for α_4. Paul Mould (unpublished results) has done similar experiments with $\alpha_5\beta_1$, using the 80 kDa fragment of fibronectin as a biotinated ligand, and there you see a very different

pattern to α_4. Whereas Ca^{2+} is slightly better than Mg^{2+} for α_4 ligands, with the 80 kDa ligand it's the other way round. I don't know whether or not there is any difference in cation specificity for different ligands for the same integrin. Current evidence suggests the cation pattern is the same.

Hogg: Martin Hemler has shown differences in the divalent cation dependency pattern for $\alpha_4\beta_1$ binding to fibronectin (Mg^{2+} only) versus VCAM-1 (Mg^{2+} and Ca^{2+}; Matsumoto & Hemler 1993).

Humphries: With isolated integrin?

Hogg: No, on the cell surface.

Humphries: You can get misleading results by concentrating just on cell adhesion assays because cation status is only one factor affecting net affinity of ligand binding; the inherent affinity of the ligand, the number of receptors and the state of receptor activation must also be taken into account.

Pober: When you did experiments studying cell attachment to the denatured collagen fragments and showed that it was an α_5-mediated effect, is that because of direct recognition of these gelatin fragments by α_5, or are the cells bringing fibronectin with them that binds to the gelatin, producing attachment through a fibronectin bridge?

Humphries: They are making fibronectin. It's well known that chondrocytic cells make huge amounts of fibronectin when they're released from the cartilage matrix. By immunofluorescence, Danny Tuckwell has observed fibronectin and α_5 in focal contacts on denatured collagen.

Pober: So it's not direct recognition of collagen by α_5 *per se*?

Humphries: No, it's a bridge. I should just add that the CB10 fragment, which is the most adhesive, is already characterized as being the major fibronectin binding site in type II collagen.

Pober: You pointed out the alternative splicing of fibronectin to either include or exclude a ligand, and you made the same comment about VCAM. I just want to throw out something for consideration about VCAM splicing. There does seem to be a profusion of molecular VCAM species in mice and rabbits. However, it's not obvious that there is alternative splicing of VCAM in humans. There is a minor species of VCAM mRNA that is present lacking exon 4, but it's always a minor species. No one has been able to find a human cell type in which there is ever more than a tiny fraction of the exon 4-deleted message, and no one has demonstrated this form of protein in a cell other than a transfectant made with the cDNA lacking exon 4.

Humphries: But it may be that those different domains in the full length molecule mediate different functions. There is some evidence for temporal differences in the use of domains I and IV during cell adhesion.

Sonnenberg: To see collagen binding you need quite high concentrations of manganese: why is this?

Humphries: You don't actually need much, but a plateau isn't reached until you use concentrations higher than 10 mM.

Sonnenberg: Is the metal binding site in the I-domain that weak?

Humphries: No, this is true for purified integrins as well. You don't normally add that amount because of complications with the assay; you certainly still get effects at sub-millimolar levels.

Elices: You mentioned that the binding of $\alpha_2\beta_1$ to collagen is conformation dependent. However, in the earlier peptide experiments, you showed that different peptides were fairly effective at inhibiting $\alpha_4\beta_1$ interaction with its ligands. How do you reconcile the apparent inconsistency that in some cases conformation seems to be important (for example, in $\alpha_2\beta_1$ binding to collagen) and in other cases it isn't.

Humphries: I think it's important in all cases. The difference between those two systems is that for the α_4 ligands the active site is in a linear peptide; the fact that you see inhibition of one by the other is because they use the same active site. With collagens, my guess is that there isn't a linear peptide that can explain that binding, so you need contributions from more than one chain and this wouldn't be possible in a fragmented molecule.

Garrod: Is anything known about α_1 binding to collagen? Are there any known parallels?

Humphries: From adhesion assays, we know that the specificity of ligand binding to the two integrins is slightly different. Overall, α_1 tends to prefer type IV collagen. $\alpha_2\beta_1$ does bind to type IV and type VI collagen, but in I-domain binding you see differences: that's why I think there may be differential use of different sites in the subunit. It will be important to test the α_1 I-domain in parallel with α_2.

References

Eble JA, Golbik R, Mann K, Kuhn K 1993 The $\alpha_1\beta_1$ integrin recognition site of the basement membrane collagen molecule [α_1(IV)$_2\alpha_2$(IV)]. EMBO (Eur Mol Biol Organ) J 12:4795–4802

Matsumoto A, Hemler ME 1993 Multiple activation states of VLA-4: mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule. J Biol Chem 268:228–234

Staatz WD, Fok KF, Zutter MM, Adams SP, Rodriguez BA, Santoro SA 1991 Identification of a tetrapeptide recognition sequence for the $\alpha_2\beta_1$ integrin in collagen. J Biol Chem 266:7363–7367

Tenchini ML, Adams JC, Gilberty C et al 1993 Evidence against a major role for integrins in calcium-dependent intercellular adhesion of epidermal keratinocytes. Cell Adhes Commun 1:35–66

General discussion III

Binding sites on the integrin $\alpha_L\beta_2$ (LFA-1) for its ligand ICAM-1

Hogg: I am going to describe work carried out by Anna Randi and Paula Stanley in my laboratory. The integrin $\alpha_L\beta_2$ (also known as lymphocyte function-associated antigen 1 [LFA-1]) has specificity for intercellular adhesion molecule 1 (ICAM-1), but it has been notably difficult to demonstrate binding of ICAM-1 to intact $\alpha_L\beta_2$ in solution. In order to identify binding sites on $\alpha_L\beta_2$ for ICAM-1, we have taken the approach of translating *in vitro* regions of the α_L subunit to obtain a 'nested' series of protein fragments with domains deleted from the N-terminus of the subunit (Fig. 1). We have then used a chimeric form of ICAM-1 with an IgG1 Fc 'tail' to precipitate these ^{35}S-methionine-labelled protein fragments. The minimum fragment with which we could obtain precipitation with ICAM-1 corresponded to domains V and VI, which are two of the three putative divalent cation-binding domains within the α subunit (i.e. domains V–VII) (Stanley et al 1994).

This result indicated that a binding site for ICAM-1 is contained within domain V and VI of the α_L subunit. In order more precisely to pinpoint the ICAM-1 binding site within this region, we next made a series of 20mer peptides covering domains IV through VII and tested their ability to inhibit T cell binding to ICAM-1 via $\alpha_L\beta_2$. The two peptides which inhibited the assay are located downstream of the putative divalent-cation binding site of domain V and overlapping the metal binding sequence of domain VI. Because domains V–VII have been speculated to resemble the classic helix-loop-helix motif of the Ca^{2+}-binding EF hand, we have modelled domain V and VI on the basis of the solved structure of the calmodulin EF hands (Stanley et al 1994). When the inhibitory peptides are positioned on the model, the two ICAM-1 binding sites lie adjacent to one another along one rim of the model and appear to form a single binding site.

When the I-domain was eliminated from the $\alpha_L\beta_2$-translated α-subunit fragments, however, there was a large decrease in the ability of ICAM-1 to precipitate $\alpha_L\beta_2$ fragments, suggesting that the I-domain is involved in binding of ICAM-1. To investigate its role in ICAM-1 binding, we expressed recombinant isolated I-domain as a fusion protein with the Fc portion of IgG1 (I-Fc) and tested a panel of anti-$\alpha_L\beta_2$ monoclonal antibodies for their ability to interact with it. The majority of the previously characterized anti-$\alpha_L\beta_2$ antibodies (18 out of 20) recognized epitopes within the I-domain (Randi & Hogg 1994). This may reflect a structural feature, e.g. this region could be more exposed and, therefore, more immunogenic than other parts of the molecule. Many of these

I-domain monoclonal antibodies block the $\alpha_L\beta_2$/ICAM-1 interaction, providing further evidence for the involvement of this domain in ligand binding. The domain could regulate binding by controlling the exposure of a cryptic binding site, or could function as a binding site itself. To test these options, we investigated the effect of recombinant $\alpha_L\beta_2$ I-domain (I-Fc) on the binding of T cells to ICAM-1 in an assay which is $\alpha_L\beta_2$/ICAM-1 dependent. I-Fc inhibited T cell binding to ICAM-1 (Fig. 2), whereas the control protein CD14-Fc had no effect on the assay; I-Fc also bound directly to immobilized recombinant ICAM-1 (Randi & Hogg 1994).

Therefore, there are at least two ICAM-1 binding sites on the α_L subunit: one in the I-domain and one in the divalent cation-binding domains V/VI. In addition, there is evidence for an RGD binding site at the N-terminus of the β_3 subunit, within an area that is highly homologous among all β-subunits (Loftus et al 1990). This suggests that the β_2 subunit may also contribute to ligand binding. How all of these sites actually work together to bind the ligand is the next question. They may act in concert, as a sort of 'maxi' ligand binding pocket, or in sequence, with the binding to one site promoting interaction with a second site. With regard to this second possibility, the notion of sequential activation arises, with the interaction of ICAM-1 with one binding site on $\alpha_L\beta_2$ leading to the exposure of a second site, resulting in more stable binding of the $\alpha_L\beta_2$/ICAM-1 receptor pair (Cabañas & Hogg 1993).

Rothlein: A lot of those antibodies against $\alpha_L\beta_2$ were screened functionally. This may be why they are directed against the I-domain, not because the I-domain is immunodominant. Have you tried blocking through the I-domain with peptides?

Hogg: We are in the process of doing that sort of experiment.

Rothlein: Do your results indicate that the I-domain has stronger binding than domains V and VI?

Hogg: Potentially, yes, but peptides from the domain V/VI region also block a T lymphocyte assay which measures $\alpha_L\beta_2$/ICAM-1 binding.

Rothlein: Do they block the purified α-subunit if you do immunoprecipitation?

Hogg: The peptides each block the T cell assay but a peptide covering the domain V site also blocks in solution. This suggests either that there is a sequential interaction between the binding sites (so if you block at any one point you block the entire interaction) or that they each critically contribute energy to the binding site (so that if you diminish one site of interaction you destabilize the whole site).

Sonnenberg: If all these proteins were made by recombinant methods in bacteria, it means that they are not glycosylated.

Hogg: The α_L protein fragments are made *in vitro* in the standard reticulocyte lysate system, and the I-domain fragment is obtained from COS cells. However, the $\alpha_L\beta_2$ I-domain protein doesn't have any *N*-linked glycosylation, nor does the domain V/VI region.

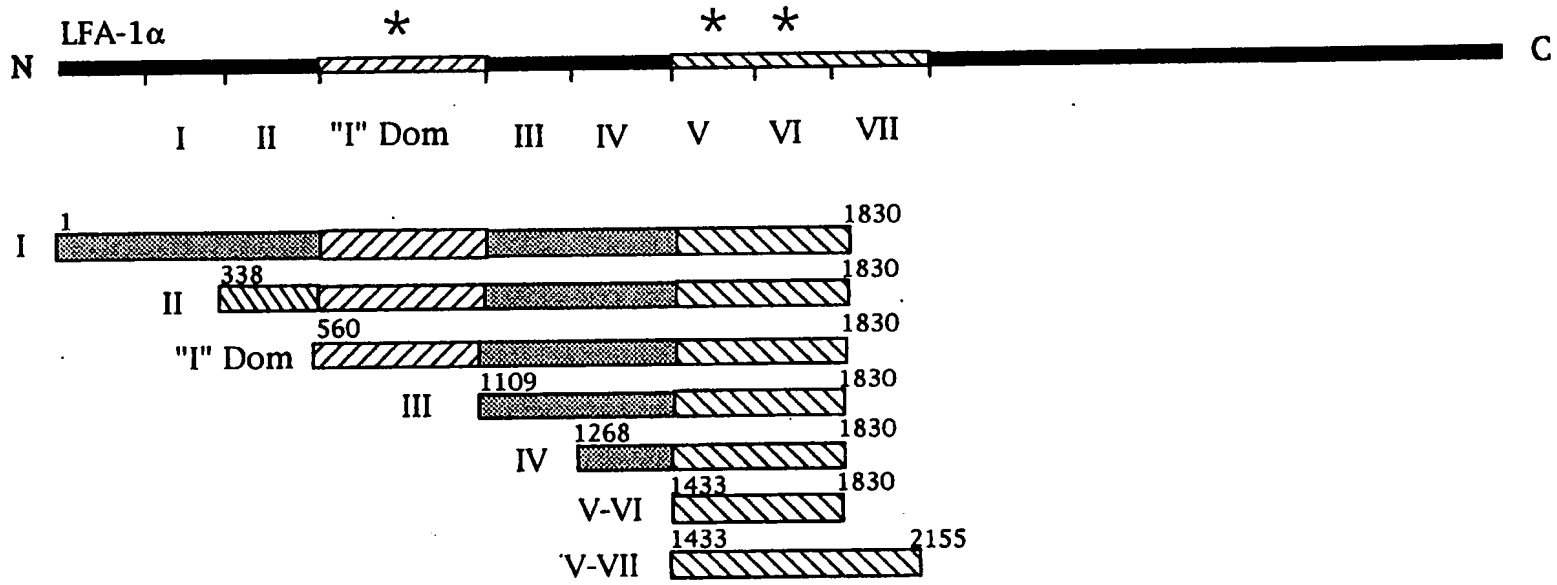


FIG. 1. The α_L (LFA-1 α-subunit) deletion series used to show binding of ICAM-1 to domains V and VI. Domains are successively deleted from the N-terminus and fragments terminate at the beginning of domain VII. The positions of ICAM-1 binding sites located on the α_L subunit are indicated by asterisks.

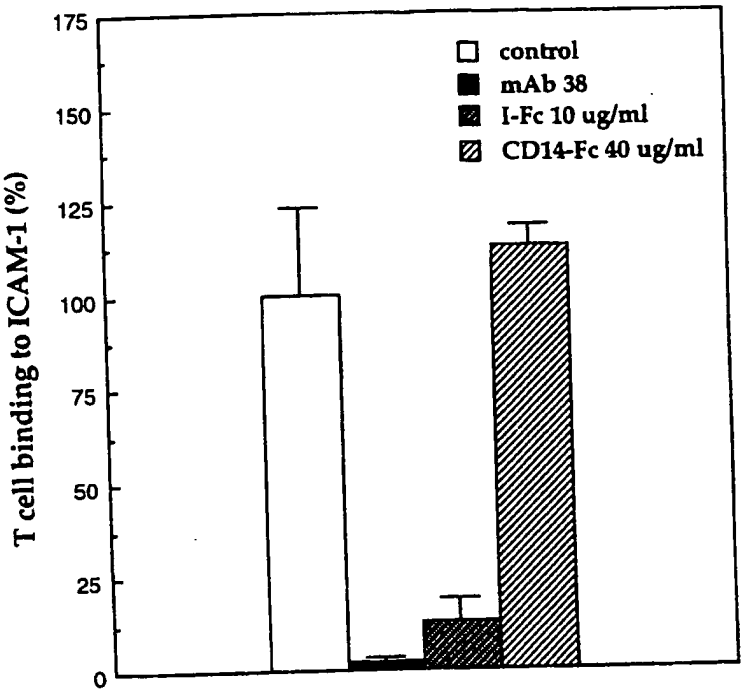


FIG. 2. T cell binding to recombinant ICAM-1-Fc. T cell binding is expressed as percentage of control binding. The inhibition of binding by a known anti-$\alpha_L\beta_2$ (LFA-1) monoclonal antibody (mAb 38) and by recombinant chimeric $\alpha_L\beta_2$ I-domain (I-Fc) is shown. The control chimeric protein CD14-Fc does not affect binding. Bars represent mean ± standard deviation of triplicates of one experiment representative of five.

Shaltiel: Is there synergism in the inhibition by the two sites?

Hogg: We haven't yet done those experiments!

Elices: Both Martin Humphries and Nancy Hogg have mentioned the importance of cation binding sites—what is the involvement of these in the binding event?

Hogg: We found that binding to the domain V and VI site, at least, was not divalent-cation dependent (Stanley et al 1994). This was unexpected and raises the question of what the role of divalent cation action might be when bound to these domains. There are lots of examples in other proteins of divalent cations acting as modulators of tertiary or quaternary structure. So it is possible that divalent cations don't affect the local secondary structure, but they might affect the relationship between the α and β subunit of an integrin. This is just a speculation for which there is no evidence at present.

Pober: Given the fact that the domain V/VI peptides do not appear to have a particular affinity for ICAM, could they be binding to $\alpha_L\beta_2$ and be perturbing its structure by competing for an internal interaction?

Hogg: That is an interesting idea. However, the fact that at least the domain V peptide can interfere with a truncated α-subunit fragment in solution does

indicate direct binding to ICAM-1 but we obviously cannot rule out the possibility that they are perturbing some other part of the structure.

Humphries: I believe the cation sites are very important, but my feeling is that they probably do more than one job. There's a lot of evidence for a role for cations in the activation of integrins as well as some very good evidence for a role in ligand binding. In addition, I think they are critical for α,β orientation. Hence, in experiments where you mutate those sites, the interpretation of the results may be complicated.

Elices: I take it from Nancy Hogg's comments that divalent cations might not be involved in the binding event *per se*. Rather, they may participate in sustaining the 3D structure.

Hogg: Or, potentially, revealing the binding site.

Humphries: You can't tell yet, there's not enough information.

References

Cabañas C, Hogg N 1993 Ligand intercellular adhesion molecule-1 has a necessary role in activation of integrin lymphocyte function-associated molecule-1. Proc Natl Acad Sci USA 90:5838–5842

Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger AL, Ginsberg MH 1990 A β_3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249:915–918

Randi AM, Hogg N 1994 I-domain of β_2 integrin lymphocyte function-associated antigen-1 contains a binding site for ligand intercellular adhesion molecule-1. J Biol Chem 269:12395–12398

Stanley P, Bates PA, Harvey J, Bennett RI, Hogg N 1994 Integrin LFA-1 α subunit contains an ICAM-1 binding site in domains V and VI. EMBO (Eur Mol Biol Organ) J 13:1790–1798

Molecular Biology of the Cell
Vol. 5, 679–690, June 1994

Regulation of Cellular Interactions with Laminin by Integrin Cytoplasmic Domains: The A and B Structural Variants of the $\alpha 6\beta 1$ Integrin Differentially Modulate the Adhesive Strength, Morphology, and Migration of Macrophages

Leslie M. Shaw and Arthur M. Mercurio

Program in Cell and Developmental Biology and Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02115

Submitted February 22, 1994; Accepted May 5, 1994
Monitoring Editor: Erkki Ruoslahti

Several integrin α subunits have structural variants that are identical in their extracellular and transmembrane domains but that differ in their cytoplasmic domains. The functional significance of these variants, however, is unknown. In the present study, we examined the possibility that the A and B variants of the $\alpha 6\beta 1$ integrin laminin receptor differ in function. For this purpose, we expressed the $\alpha 6$A and $\alpha 6$B cDNAs, as well as a truncated $\alpha 6$ cDNA ($\alpha 6$-ΔCYT) in which the cytoplasmic domain sequence was deleted after the GFFKR pentapeptide, in P388D$_1$ cells, an $\alpha 6$ deficient macrophage cell line. Populations of stable $\alpha 6$A, $\alpha 6$B, and $\alpha 6$-ΔCYT transfectants that expressed equivalent levels of cell surface $\alpha 6$ were obtained by fluorescence-activated cell sorter and shown to form heterodimers with endogenous $\beta 1$ subunits. Upon attachment to laminin, the $\alpha 6$A transfectants extended numerous pseudopodia. In contrast, the $\alpha 6$B transfectants remained rounded and extended few processes. The transfectants were also examined for their ability to migrate toward a laminin substratum using Transwell chambers. The $\alpha 6$A transfectants were three- to fourfold more migratory than the $\alpha 6$B transfectants. The $\alpha 6$-ΔCYT transfectants did not attach to laminin in normal culture medium, but they did attach in the presence of Mn^{2+}. The $\alpha 6$-ΔCYT transfectants migrated to a lesser extent than either the $\alpha 6$A or $\alpha 6$B transfectants in the presence of Mn^{2+}. The $\alpha 6$ transfectants differed significantly in the concentration of substratum bound laminin required for half-maximal adhesion in the presence of Mn^{2+}:$\alpha 6$A (2.1 μg/ml), $\alpha 6$B (6.3 μg/ml), and $\alpha 6$-ΔCYT (8.8 μg/ml). Divalent cation titration studies revealed that these transfectants also differed significantly in both the [Ca^{2+}] and [Mn^{2+}] required to obtain half-maximal adhesion to laminin. These data demonstrate that the A and B variants of the $\alpha 6$ cytoplasmic domain can differentially modulate the function of the $\alpha 6\beta 1$ extracellular domain.

INTRODUCTION

The role of integrin adhesion receptors as conduits for signaling information in and out of cells has been established in recent years (for review see Ginsberg *et al.*, 1992; Hynes, 1992; Sastry and Horwitz, 1993; Schwartz, 1993). Although the mechanisms of integrin signaling are poorly understood, one insightful set of findings is that integrin cytoplasmic domains play important regulatory roles in both inside-out and outside-in signaling pathways (reviewed in Sastry and Horwitz, 1993). This conclusion has been derived largely from mutational analyses of integrin cytoplasmic domains. Such studies have shown that the $\beta 1$ integrin cytoplasmic domain provides a critical linkage with the cytoskeleton that is essential for adhesion (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992). Similar studies have indicated that integrin α subunits

influence both receptor function and subsequent post-ligand binding events such as focal contact formation, migration, and gel contraction (Hibbs *et al.*, 1991; O'Toole *et al.*, 1991; Bauer *et al.*, 1993; Briesewitz *et al.*, 1993; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993; Ylanne *et al.*, 1993). One key problem that is highlighted by these observations is how information in integrin cytoplasmic domains is transmitted to both integrin extracellular domains and to signaling molecules inside the cell.

Functional analyses of integrin cytoplasmic domains must consider the finding that many integrin subunits have structural variants that are identical in their extracellular and transmembrane domains but that differ in their cytoplasmic domains. This group includes the $\beta 1$ (Altruda *et al.*, 1990; Languino and Ruoslahti, 1992), $\beta 3$ (van Kuppevelt *et al.*, 1989), $\beta 4$ (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990; Tamura *et al.*, 1990; Clarke and Mercurio, 1993), $\alpha 3$ (Tamura *et al.*, 1991), $\alpha 6$ (Hogervorst *et al.*, 1991; Tamura *et al.*, 1991), and $\alpha 7$ (Collo *et al.*, 1993) subunits. At least two structural variants exist for each of these integrin subunits. To date, most studies on integrin cytoplasmic domain variants have not focused directly on function but have examined such things as their relative patterns of expression. For example, the A and B variants of both the $\alpha 6$ and $\alpha 7$ integrin subunits exhibit developmentally regulated expression patterns (Cooper *et al.*, 1991; Collo *et al.*, 1993; Ziober *et al.*, 1993). The central issue that now needs to be addressed is whether cytoplasmic domain variants of the same integrin exhibit differences in function.

We have focused our efforts on the function and regulation of the $\alpha 6$A and $\alpha 6$B cytoplasmic domain variants. This work has involved the use of macrophages because adhesion to laminin is mediated by the $\alpha 6\beta 1$ integrin in these cells and this adhesion is markedly increased in response to inside-out signals (Mercurio and Shaw, 1988; Shaw *et al.*, 1990). Expression of the $\alpha 6$A and $\alpha 6$B cDNAs in an $\alpha 6$-deficient macrophage cell line, P388D$_1$, enabled us to determine that both the $\alpha 6A\beta 1$ and $\alpha 6B\beta 1$ integrin variants can be activated by inside-out signaling pathways (Shaw *et al.*, 1993). A truncated $\alpha 6$ cDNA, $\alpha 6$-ΔCYT, was constructed in which the human cytoplasmic domain sequence was deleted after the GFFKR pentapeptide. Expression of this cDNA in P388D$_1$ cells resulted in the surface expression of a chimeric $\alpha 6$-$\Delta CYT\beta 1$ integrin that was unable to mediate laminin adhesion or increase this adhesion in response to phorbol 12-myristate 13-acetate (PMA) (Shaw and Mercurio, 1993). The $\alpha 6$A-ΔCYT transfectants adhered to laminin, however, when extracellular Ca^{2+}/Mg^{2+} was replaced with Mn^{2+}. A logical question that emerged from these studies is whether the $\alpha 6A\beta 1$ and $\alpha 6B\beta 1$ variants, as well as the $\alpha 6$-$\Delta CYT\beta 1$ mutant, exhibit differences in their ability to initiate specific cellular functions subsequent to laminin attachment.

In the present study, we observed that P388D$_1$ cells transfected with either the human $\alpha 6$A, $\alpha 6$B, or $\alpha 6$-ΔCYT integrin cDNAs differed markedly in their morphology on laminin and in their ability to migrate toward a laminin gradient. Subsequent analysis of these $\alpha 6$ transfectants using a combination of laminin and divalent cation titrations revealed that they differed in their relative adhesive strength for laminin. Specifically, we found the following order of relative adhesive strengths: $\alpha 6A\beta 1 \gg \alpha 6B\beta 1 > \alpha 6\Delta CYT\beta 1$. These data suggest that specific sequences within the $\alpha 6$A and $\alpha 6$B cytoplasmic domains differentially modulate the functional activity of the $\alpha 6\beta 1$ extracellular domain.

MATERIALS AND METHODS

Cells

The P388D$_1$ mouse macrophage cell line was obtained from the American Type Tissue Collection (Rockville, MD). Cells were maintained in RPMI containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (RPMI-H) and 15% certified fetal bovine serum (GIBCO, Grand Island, NY).

cDNA Transfections

The human $\alpha 6$A and $\alpha 6$B cDNAs were cloned by polymerase chain reaction and subcloned into the eukaryotic expression vector pRc/CMV as described previously (Shaw *et al.*, 1993). The $\alpha 6$-ΔCYT mutant cDNA was constructed as described previously (Shaw and Mercurio, 1993). The pRc/CMV vectors containing either the $\alpha 6$A, $\alpha 6$B, or $\alpha 6$-ΔCYT cDNAs, as well as the vector alone, were transfected into the P388D$_1$ cell line with Lipofectin (GIBCO). Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.4 mg/ml) (GIBCO). The stable transfectants were pooled and populations of cells that expressed the human $\alpha 6$ subunits on the cell surface were isolated by fluorescence-activated cell sorter (FACS). A human $\alpha 6$ integrin specific monoclonal antibody (mAb), 2B7, was used for this sorting and for subsequent analysis of the transfectants (Shaw *et al.*, 1993). The sorting was repeated sequentially for each transfectant to enrich for homogeneous populations of cells expressing equivalent levels of the transfected $\alpha 6$ subunits on the cell surface.

Flow Cytometry

Transfected P388D$_1$ cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin (PBS/BSA). Aliquots of cells (3×10^5) were incubated for 30 min at 4°C with PBS/BSA containing murine IgG Fc fragment (6 μg/ml) (Jackson Immunoresearch, Avondale, PA). The mAb 2B7 was added at a concentration of 2 μg/ml, and the cells were incubated for an additional hour at 4°C. The cells were washed three times with PBS/BSA and then incubated with goat F(ab')$_2$ anti-mouse IgG coupled to fluorescein (Tago, Burlingame, CA) for 1 h at 4°C. After washing three times with PBS/BSA, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson, Lincoln Park, NJ).

Adhesion Assays

Adhesion assays were performed as described previously (Shaw and Mercurio, 1993). Briefly, multiwell tissue culture plates (11.3 mm diameter) were coated overnight at 4°C with 0.2 ml of PBS containing murine Englebreth-Holm-Swarm (EHS) laminin at the concentrations indicated in the individual figure legends. Laminin was purified from

the EHS sarcoma as described (Kleinman *et al.*, 1982). The wells were then washed with PBS and 10^5 cells in Puck's Saline A (200 μl) (Sigma, St. Louis, MO) containing 25 mM HEPES, and divalent cations were added to each well. For the laminin titration adhesion assays, 96-well nontissue culture-treated plates (Falcon, Lincoln Park, NJ) were used, and the wells were counter-coated for 3 h at room temperature with Puck's Saline A containing 1% BSA. BSA was also included in the incubation buffer during the laminin titration adhesion assays. After a 45-min incubation at 37°C, the wells were washed three times with Puck's Saline A at 37°C, fixed for 15 min with methanol, and stained with a 0.2% solution of crystal violet in 2% ethanol. The crystal violet stain was solubilized with a 1% solution of sodium dodecyl sulfate, and adhesion was quantitated by measuring the absorbance at 600 nm. For photomicrographs, the adherent cells were fixed in RPMI-H containing 3.7% formaldehyde for 15 min, permeabilized with methanol for 10 min, and then stained with crystal violet.

Migration Assays

Cell migration assays were performed using 6.5-mm Transwell chambers (8 μm pore size) (CoStar, Cambridge, MA). RPMI-H containing 15 μg/ml laminin (0.6 ml) or Puck's Saline A containing 25 mM HEPES, 0.5 mM $MnCl_2$, 0.1 mM $CaCl_2$, 1 mM L-Glutamine, and 15 μg/ml laminin were added to the bottom well, and the filters were coated for ~30 min at 37°C. Cells were resuspended in the appropriate buffer at a concentration of 10^6/ml, and 10^5 cells were added to the top well of the Transwell chambers. After a 24-h incubation, the cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of crystal violet in 2% ethanol. Migration was quantitated by counting using brightfield optics with a Nikon Diaphot microscope (Garden City, NY) equipped with a 16-square reticle. The surface area of this grid was determined to be 1 mm^2. Five separate fields were counted for each filter.

To examine the Ca^{2+} requirements for cell migration, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added to the RPMI-H at a concentration of 0.5 mM and the $MgCl_2$ concentration was increased to 2 mM. 2B7 was included in some assays at a concentration of 8 μg/ml to examine inhibition of migration.

RESULTS

In the studies described in this report, $P388D_1$ cells were used that had been transfected with either the human α6A, α6B, or mutant α6-ΔCYT integrin cDNAs. Populations of cells that expressed equivalent levels of cell surface α6 were obtained by FACS using 2B7, a mAb specific for the human α6 integrin subunit (Figure 1). The levels of α6 expression on the cell surface of the transfectants were monitored by FACS analysis frequently, and only populations that expressed equivalents levels were used for comparative experiments. The α6A, α6B, and α6-ΔCYT subunits formed heterodimers with endogenous β1 subunits (Shaw and Mercurio, 1993).

The α6A and α6B Transfectants Differ in Their Morphology on Laminin

Both the α6A and α6B cDNAs are capable of restoring the ability of $P388D_1$ cells to adhere to a laminin substratum (Shaw *et al.*, 1993). In the present study, however, we observed that these two populations of transfectants differed markedly in their morphology after attachment to a laminin substratum (Figure 2). Specifically, 68% of the α6A transfectants extended pseudopodia compared to 20% of the α6B transfectants. In addition, the pseudopodia extended by the α6A transfectants were considerably longer than those extended by the α6B transfectants. The α6B transfectants exhibited a more rounded appearance than the α6A transfectants. This difference in morphology is specific to laminin because these transfectants exhibited a similar morphology when attached to a fibronectin substratum (Figure 2).

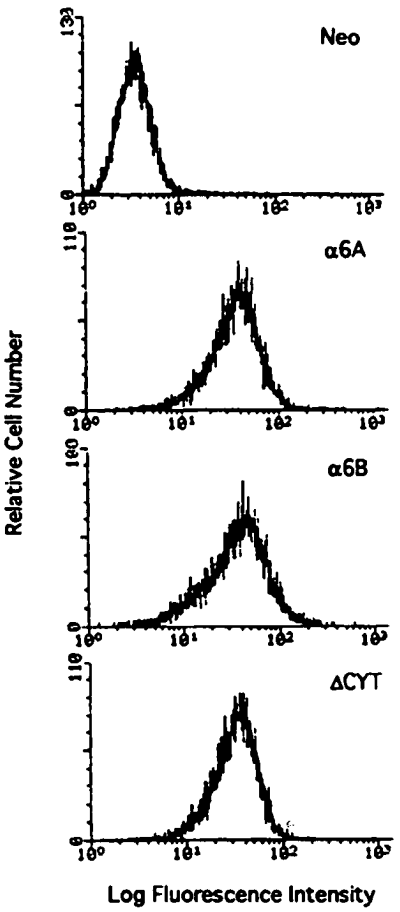


Figure 1. Surface expression of the human α6A, α6B, and α6-ΔCYT integrin subunits in $P388D_1$ transfectants. Populations of transfected $P388D_1$ cells expressing either the α6A, α6B, or α6-ΔCYT subunits on the cell surface were isolated by sequential FACS using 2B7, a mAb specific for the human α6 integrin subunit, and then analyzed by flow cytometry. Neo, $P388D_1$ cells transfected with the vector alone.

The α6A and α6B Transfectants Differ in Their Migration Toward Laminin

Extensive pseudopod formation is characteristic of motile cells (Trinkaus, 1984). For this reason, the α6A and α6B transfectants were examined for their ability to migrate toward a laminin substratum using Transwell

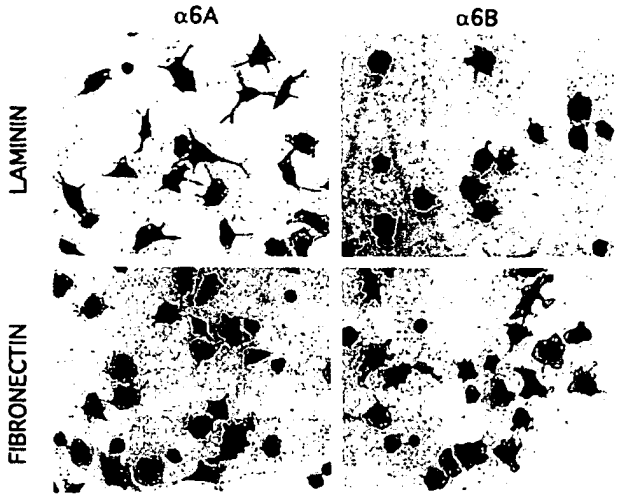


Figure 2. Photomicrographs of adherent P388D$_1$ transfectants. α6A-P388D$_1$ and α6B-P388D$_1$ transfectants were allowed to adhere to either laminin or fibronectin substrata for 45 min. After washing, adherent cells were fixed and stained as described in MATERIALS AND METHODS. Stained cells were photographed using brightfield optics. Magnification, ×2000.

chambers. These migration assays were performed in the same medium, RPMI-H, that had been used to examine their adhesion and morphology. The results obtained indicated that the α6A and α6B transfectants differed significantly ($p < 0.01$) in their ability to migrate toward a laminin gradient. As shown in Figure 3A, the α6A transfectants were three- to fourfold more migratory toward laminin than the α6B transfectants in a 24-h assay.

PMA did not increase the number of either the α6A or α6B transfectants that migrated toward laminin (Figure 3A). In fact, a slight decrease in the number of cells that migrated was often observed with PMA. This finding is in contrast to the marked increase in adhesion of these transfectants to laminin in response to PMA stimulation that we previously reported (Shaw *et al.*, 1993). The migration of both the α6A and α6B transfectants is α6β1 dependent because it was completely inhibited by 2B7 under all of the conditions examined (Figure 3A). P388D$_1$ cells that were transfected with the α6 cytoplasmic deletion mutant, α6-ΔCYT, did not migrate toward laminin under these conditions. This observation is consistent with our previous finding that the α6-ΔCYT transfectants did not adhere to laminin in RPMI-H (Shaw and Mercurio, 1993). P388D$_1$ cells transfected with the pRc/CMV vector alone also did not migrate (Figure 3A).

The difference in migration that was observed for the transfectants was specific for laminin because the α6A, α6B, and α6-ΔCYT transfectants migrated to the same extent when fetal calf serum (15%) was included in the bottom well of the Transwell chamber (Figure 3B). It is interesting to note that these cells did not migrate toward fibronectin or collagen I in this assay. Presumably, the migration in serum refects their ability to migrate on vitronectin.

The α6 Transfectants Differ in Their Relative Binding Strengths for Laminin

One hypothesis that can be formulated to explain the data presented in the preceding sections is that the α6A and α6B transfectants differ in their relative adhesive strength for laminin. Such differences in adhesive strength can be detected by measuring the attachment of the transfectants as a function of increasing laminin concentration. Recent quantitative studies have demonstrated that the slope of the relationship between the strength of cell substratum attachment and the concentration of matrix protein increases with both increasing adhesion receptor number and affinity between adhesion receptor and matrix protein (Cozen-Roberts *et al.*, 1990; DiMilla *et al.*, 1993). In our previous adhesion assays, we had used saturating laminin concentrations (20 μg/ml) to coat the microtiter wells, and the possibility existed that differences in binding may not have been apparent at this concentration.

To determine if the α6 transfectants differed in their relative adhesive strength for laminin, adhesion assays were performed using a range of laminin substratum concentrations. The assays were carried out using conditions that promote maximal adhesion (i.e., 0.5 mM Mn^{2+}) to discount the possibility that any observed differences could be attributed to differences in the response of these transfectants to inside-out signals. In addition, because the α6-ΔCYT transfectants can adhere to laminin in the presence of this cation, they could be examined in this assay as well (Shaw and Mercurio, 1993). Populations of α6 transfectants were used that expressed equivalent levels of α6 on the cell surface (Figure 1) to exclude the possibility that any differences in adhesion could be attributed to differences in receptor number. At the highest concentration of laminin used (20 μg/ml), all three of the transfectant populations adhered at equivalent levels. However, the transfectants differed in the concentration of laminin that was required for half-maximal adhesion (Figure 4A and Table 1). The α6A transfectants exhibited the strongest adhesion with half-maximal adhesion occurring at a concentration of ~2.1 μg/ml of laminin. The α6B transfectants required 6.3 μg/ml of laminin for half-maximal adhesion, and the α6-ΔCYT transfectants exhibited the weakest adhesion with a half-maximal requirement of 8.8 μg/ml of laminin. The differences in the concentration of laminin required for half-maximal adhesion of the α6A and α6B transfectants are significant ($p < 0.05$) (Table 1). These data indicate that the α6 transfectants differ in their relative adhesive strength for laminin.

In response to PMA activation, the relative adhesive strength of the α6A transfectants increased only slightly

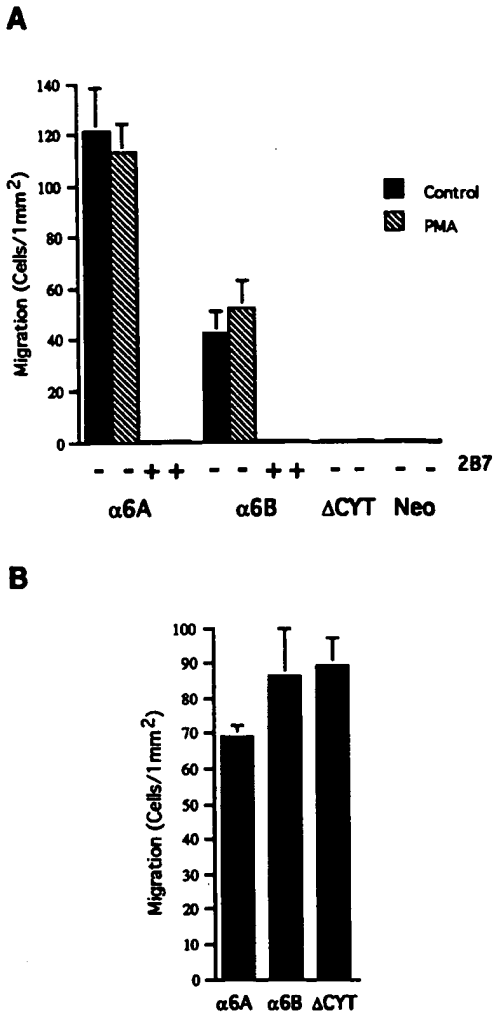


Figure 3. (A) Migration of α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ cells toward a laminin substratum. RPMI-H containing laminin (15 μg/ml) was added to the bottom well of a Transwell chamber (8 μM pore filters), and 10^5 cells were added to the top well. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Antibodies specific for α6 (8 μg/ml) were included in the assays as indicated (±2B7). Migration was quantitated by counting. The data shown are the mean values (±SD) of two separate experiments done in duplicate. (B) Migration of α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ cells toward fetal calf serum. RPMI-H containing serum (15%) was added to the bottom well of a Transwell chamber, and assays were performed as described for laminin. The data shown are the mean values (±SEM) of one experiment done in duplicate. 2B7, α6-specific mAb; Neo, P388D$_1$ cells transfected with the expression vector alone; ■ control transfectants; ▨, PMA-stimulated transfectants.

(Figure 4B). However, the concentration of laminin required for half-maximal adhesion of both the α6B and α6-ΔCYT transfectants decreased markedly to ~2 μg/ml, a value similar to that observed for the α6A transfectants (Figure 4B). Thus, all of the transfectants exhibited similar adhesive strengths for laminin in response to PMA. The behavior of the α6-ΔCYT transfectants in this experiment does not conflict with our previous conclusion that this mutant cannot respond to PMA because in the present experiment adhesion was induced by the presence of Mn^{2+}. Most likely, other targets of PMA such as cytoskeletal proteins strengthen Mn^{2+}-induced adhesion of these cells.

Ca^{2+} Is Required for α6-mediated Migration but Not Adhesion

Because integrin heterodimers can differ in their Ca^{2+} requirements for adhesion and migration (Grzesiak *et al.*, 1992; Leavesley *et al.*, 1993), we thought it important to examine the role of Ca^{2+} in the behavior of the α6A and α6B transfectants on laminin. For this purpose, both adhesion and migration assays were performed in the presence of 0.5 mM EGTA to chelate extracellular Ca^{2+}.

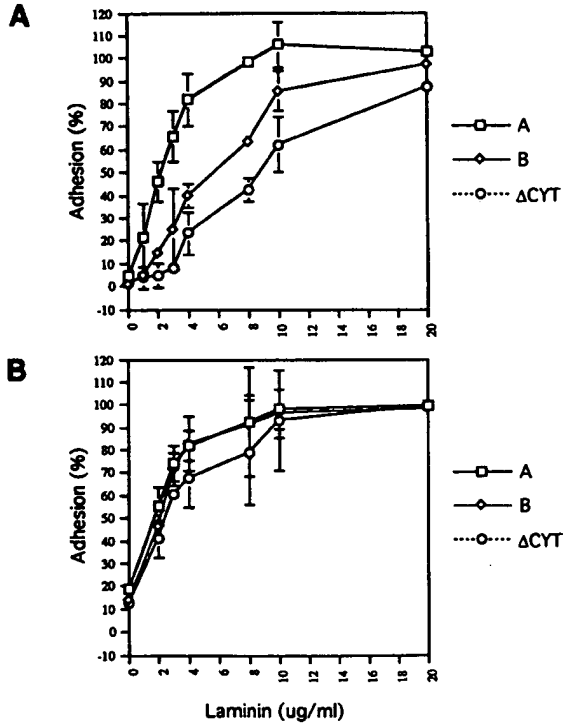


Figure 4. Laminin titration adhesion assays. (A) α6A-P388D$_1$, α6B-P388D$_1$, and α6ΔCYT-P388D$_1$ transfectants were assayed for their ability to adhere to a range of laminin substratum concentrations. Tissue culture wells were coated with EHS laminin (1–20 μg/ml) overnight and counter-coated with 1% BSA for several hours. Transfected cells (5×10^4) were resuspended in Puck's Saline A containing 1% BSA and 0.5 mM Mn^{2+} and added to the protein-coated wells. After 45 min at 37°C, nonadherent cells were removed by washing, and adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. (B) The adhesion assays were performed in the presence of PMA (50 ng/ml). The data shown are the mean values (±SD) from two experiments done in triplicate.

EGTA did not affect the viability of the cells (unpublished observation). The results obtained indicate that Ca^{2+} is not required for adhesion of either the α6A or α6B transfectants to a laminin substratum (Figure 5A). In fact, chelation of Ca^{2+} enhances the constitutive adhesion of both the α6A and α6B transfectants. This finding suggests that Ca^{2+} is a negative regulator of α6β1 function, a conclusion we deduced previously for peritoneal macrophages (Shaw and Mercurio, 1993). In contrast to their adhesion, however, no migration toward laminin was observed for either the α6A or α6B transfectants when extracellular Ca^{2+} was chelated (Figure 5B). Similar to the data shown in Figure 3, a three- to fourfold difference in the migration of the α6A and α6B transfectants was evident in the presence of Ca^{2+} (Figure 5B).

The α6 Transfectants Differ in Their Cation Sensitivity for Adhesion to Laminin

Extracellular Mn^{2+} (150 μM) markedly increases the ability of the α6A-, α6B-, and α6-ΔCYT P388D$_1$ transfectants to adhere to laminin (Shaw and Mercurio, 1993). Presumably, the interaction of Mn^{2+} with divalent cation binding sites in the α6 extracellular domain increases the affinity of the α6β1 integrin for laminin (Sonnenberg *et al.*, 1988; Shaw and Mercurio, 1991). In contrast, extracellular Ca^{2+} negatively regulates the ability of these α6 transfectants to bind laminin (Figure 5). Based on these observations, we reasoned that adhesion of the transfectants to laminin at a fixed Mn^{2+} concentration could be inhibited by increasing concentrations of Ca^{2+}. Moreover, differences in the relative affinities of the α6 transfectants for Ca^{2+} may be apparent in such an assay. As shown in Figure 6A, the α6A, α6B, and α6-ΔCYT transfectants exhibited the same amount of adhesion in the presence of 0.5 mM Mn^{2+} and the absence of Ca^{2+}. However, these transfectants did differ in the [Ca^{2+}] required to obtain half-maximal adhesion in the presence of 0.5 mM Mn^{2+}. Specifically, the α6A transfectants exhibited half-maximal adhesion at a [Ca^{2+}] of 5.5 mM, the α6B transfectants at a [Ca^{2+}] of 1.8 mM, and the α6-ΔCYT transfectants at a [Ca^{2+}] of 0.8 mM (Figure 6B). The difference in the [Ca^{2+}] required for half-maximal adhesion of the α6A and α6B transfectants is significant ($p < 0.05$) (Table 1). In the presence of PMA, the α6A and α6B transfectants were both resistant to Ca^{2+} titration at the concentrations examined. However, although PMA increased the resistance of the α6-ΔCYT transfectants to Ca^{2+} inhibition, they still exhibited sensitivity to Ca^{2+} under the conditions examined (Figure 6C).

In addition to the Ca^{2+} titration experiment, we also performed a Mn^{2+} titration to determine the concentration of Mn^{2+} required for half-maximal adhesion of each of the transfectants. All three transfectant populations adhered to laminin to the same extent at the maximal [Mn^{2+}] examined, 0.5 mM Mn^{2+} (Figure 6A). Similar to the Ca^{2+} titration data, the α6A, α6B, and α6-ΔCYT transfectants differed in the [Mn^{2+}] required to obtain half-maximal adhesion. The α6A transfectants exhibited half-maximal adhesion at a [Mn^{2+}] of 14.7 μM, the α6B transfectants at a [Mn^{2+}] of 33.5 μM, and the α6-ΔCYT transfectants at a [Mn^{2+}] of 39.0 μM (Figure 7). The differences in the [Mn^{2+}] required for half-maximal adhesion of the α6A and α6B transfectants and the α6B and α6-ΔCYT transfectants are significant ($p < 0.05$) (Table 1).

Analysis of α6-ΔCYT Migration

The ability of the α6-ΔCYT transfectants to migrate in normal culture medium cannot be assayed because they do not attach to laminin in the presence of physiological concentrations of Ca^{2+} and Mg^{2+}. Although these mutant transfectants adhere to laminin in the presence of Mn^{2+} (Shaw and Mercurio, 1993), the use of this cation is complicated by the fact that Ca^{2+} is required for migration (Figure 5), and Ca^{2+} negatively regulates Mn^{2+} adhesion (Figure 6). However, it became apparent from the data in Figure 6 that at divalent cation concentrations sufficient to promote migration (0.5 mM Mn^{2+} and 0.1 mM Ca^{2+}), the α6-ΔCYT transfectants adhered to laminin as well as the α6A and α6B transfectants. Under these conditions, the α6A transfectants were two- to threefold more migratory than the α6B transfectants (Figure 8), a difference similar to that observed in RPMI-

Table 1. Summary of laminin and divalent cation titration data

	Control	PMA
[Laminin] required for half-maximal adhesion (μg/ml)		
α6A	2.1 ± 0.4	1.8 ± 0.3
α6B	6.3 ± 0.3[a]	2.0 ± 0.2
ΔCYT	8.8 ± 0.6[b]	2.2 ± 0.8
[Ca^{2+}] required for half-maximal adhesion (mM)		
α6A	5.5 ± 0.4	>10
α6B	1.8 ± 0.3[a]	>10
ΔCYT	0.8 ± 0.3	8.1 ± 1.9
[Mn^{2+}] required for half-maximal adhesion (mM)		
α6A	14.7 ± 2.9	N.D.
α6B	33.5 ± 0.5[a]	N.D.
ΔCYT	39.0 ± 1.0[b]	N.D.

Mean values values (±SEM) for the [laminin], [Ca^{2+}], and [Mn^{2+}] required for half-maximal adhesion of the α6 transfectants to laminin were determined from the data shown in figures 4, 6, and 7. Statistical differences between pairs of transfectants were determined using Student's *t* test.

[a] $p < 0.05$ in comparison to α6A.

[b] $p < 0.05$ in comparison to α6B.

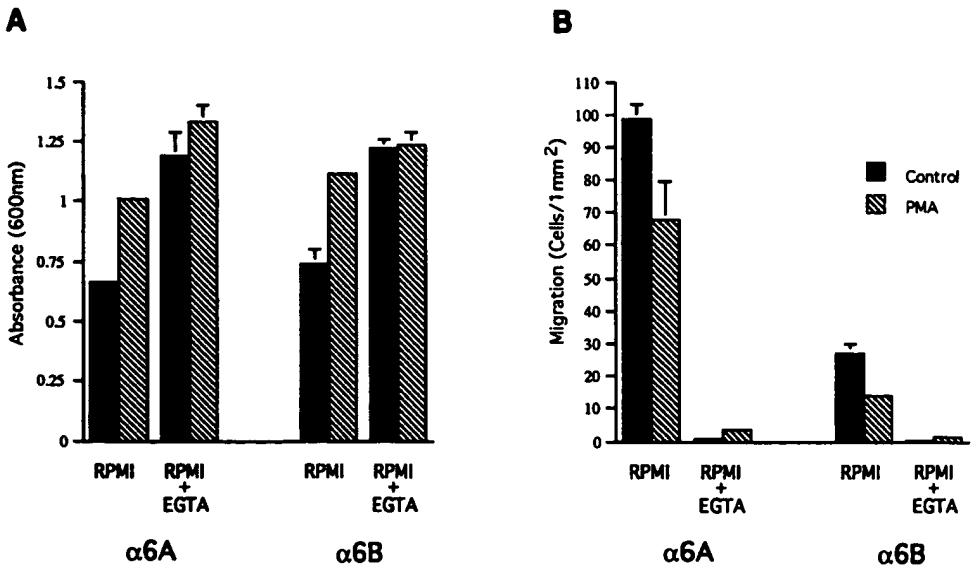


Figure 5. Ca^{2+} requirements for $\alpha 6\beta 1$ dependent adhesion and migration. (A) α6A-P388D$_1$ and α6B-P388D$_1$ transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg^{2+} and added to laminin-coated wells at a concentration of 10^5 cells per well. PMA (50 ng/ml) was added to some of the wells, and the multiwell plates were incubated for 45 min at 37°C. After washing the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SD) from a representative experiment done in triplicate. (B) α6A-P388D$_1$ and α6B-P388D$_1$ transfectants were resuspended in RPMI-H or RPMI-H containing 0.5 mM EGTA and 2 mM Mg^{2+} and added to the top well of a Transwell chamber. RPMI-H containing laminin (15 μg/ml) was added to the bottom wells. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Migration was quantitated by counting. The data shown are the mean values (±SD) from a representative experiment done in duplicate. Neo, P388D$_1$ cells transfected with the vector alone; ■, control transfectants; ▨, PMA-stimulated transfectants.

H (Figure 3). Interestingly, the α6-ΔCYT transfectants exhibited some migration toward laminin in the presence of these divalent cations, but the amount of this migration was low in comparison to that observed for the α6A and α6B transfectants (Figure 8). The differences that were observed for the migration of the α6A and α6B transfectants under both control and PMA conditions are significant ($p < 0.01$). The differences in migration between the α6B and α6-ΔCYT transfectants were not found to be significant ($p > 0.05$).

DISCUSSION

The finding that multiple cytoplasmic domain variants exist for several integrin subunits has suggested that such variants differ in function. We have studied this possibility using the two known structural variants of the α6 integrin, α6A and α6B. For this purpose, we used P388D$_1$ cells that had been transfected with either the α6A or α6B cDNAs and assessed their morphology on a laminin substratum and their ability to migrate toward a laminin gradient. The results obtained indicate that the α6A transfectants extend considerably more pseudopodia on laminin and are markedly better at migrating toward laminin than the α6B transfectants. Further investigation into the possible mechanism(s) responsible for these differences revealed that the α6A and α6B transfectants differ in their adhesive strength for laminin. The α6A and α6B transfectants also exhibited relative differences in their divalent cation requirements for adhesion. Taken together, these data indicate that sequences within the α6A and α6B cytoplasmic domains can differentially modulate the extracellular ligand and cation binding domains of the $\alpha 6A\beta 1$ and $\alpha 6B\beta 1$ receptors.

The importance of α subunit cytoplasmic domains in regulating integrin function has been established by several labs including ours (Hibbs *et al.*, 1991; O'Toole *et al.*, 1991; Bauer *et al.*, 1993; Briesewitz *et al.*, 1993; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993; Ylanne *et al.*, 1993). Deletion of the α2, α4, and α6 cytoplasmic domains after the highly conserved GFFKR sequence abolished the ability of the mutant $\alpha 2\beta 1$, $\alpha 4\beta 1$, and $\alpha 6\beta 1$ integrins to mediate constitutive adhesion to their respective ligands and to augment this adhesion in response to inside-out signals (Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993). Deletion of other α subunit cytoplasmic domains either increased (αIIb [O'Toole *et al.*, 1991]) or had no effect (αL, α1, α5 [Hibbs *et al.*, 1991; Bauer *et al.*, 1993; Briesewitz *et al.*, 1993; Ylanne *et al.*, 1993]) on receptor activity, but some of these differences may be attributed to the fact that not all of these deletions were made at the same position in the cytoplasmic domain (Hibbs *et al.*, 1991; O'Toole *et al.*, 1991). The data we have obtained in this study indicate that the α6 subunit cytoplasmic domain not only is necessary for regulating integrin ligand binding function but that this regulation is sequence specific. This conclusion differs from that obtained in recent studies that used chimeric integrin subunits, which differed only in their α subunit cytoplasmic domains, to

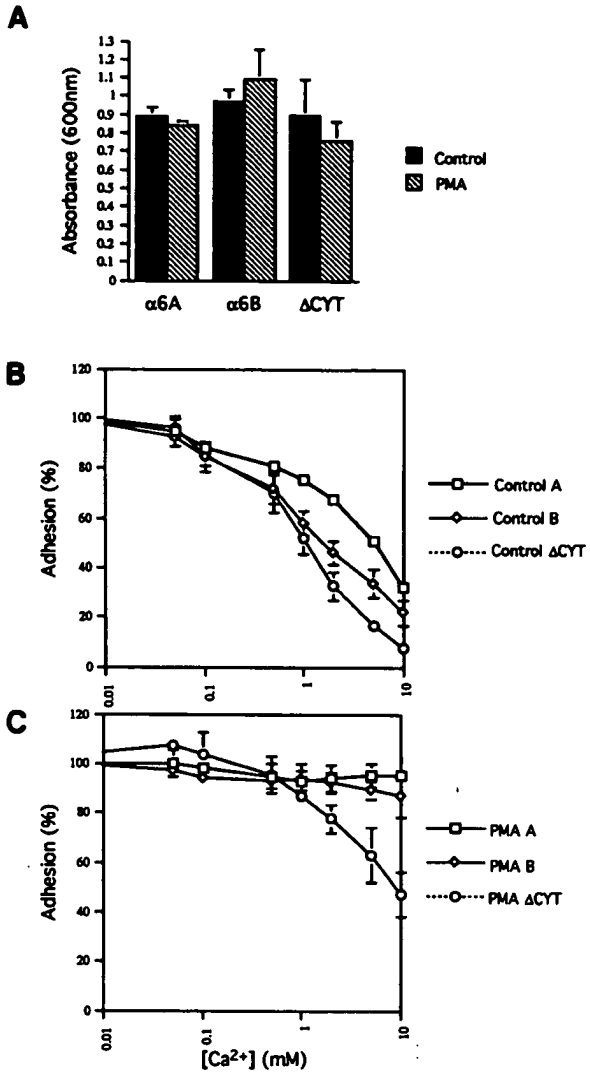


Figure 6. Ca^{2+} titration adhesion assays. Tissue culture wells were coated overnight with 20 μg/ml laminin. α6A-P388D_1, α6B-P388D_1, and α6ΔCYT-P388D_1 transfectants were resuspended in Puck's Saline A containing 0.5 mM Mn^{2+} and Ca^{2+} (0–10 mM) and added to the protein-coated wells at a concentration of 10^5 cells per well. PMA (50 ng/ml) was added to some of the wells, and the multiwell plates were incubated for 45 min at 37°C. After washing, the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SEM) from three experiments done in duplicate. (A) Adhesion in the presence of 0.5 mM Mn^{2+}; (B) adhesion in the presence of 0.5 mM Mn^{2+} and increasing [Ca^{2+}]; (C) same conditions as in B but in the presence of PMA. ■, control transfectants; ▨, PMA-stimulated transfectants.

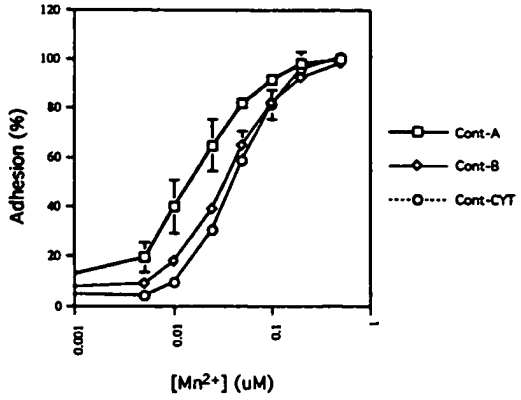


Figure 7. Mn^{2+} titration adhesion assays. Tissue culture wells were coated overnight with 20 μg/ml laminin. α6A-P388D_1, α6B-P388D_1, and α6ΔCYT-P388D_1 transfectants were resuspended in Puck's Saline A containing Mn^{2+} (0–1000 μM) and added to the protein-coated wells at a concentration of 10^5 cells per well. The multiwell plates were incubated for 45 min at 37°C. After washing the adherent cells were fixed, stained, and quantitated as described in MATERIALS AND METHODS. The data shown are the mean values (±SEM) from two representative experiments done in duplicate.

assess the contribution of these domains to integrin activity (Chan *et al.*, 1992; Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993). Although these chimeric subunits exhibited different abilities to mediate migration and gel contraction (Chan *et al.*, 1992), they did not differ in their apparent ligand binding activity. Thus, the conclusion was drawn that the cytoplasmic domain of the α subunit is necessary for regulating receptor ligand binding but that the specific sequence of the α subunit cytoplasmic domain is not critical for this regulation. In contrast, our data demonstrate that sequence differences between the α6A and α6B variants account for differences in the adhesive strength, morphology,

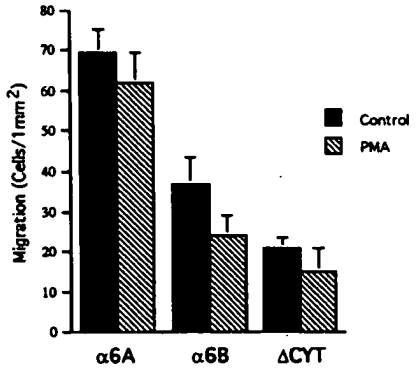


Figure 8. Effect of Mn^{2+} on the migration of the α6A-P388D_1, α6B-P388D_1, and α6ΔCYT-P388D_1 transfectants toward laminin. Puck's Saline A containing 0.5 mM Mn^{2+}, 0.1 mM Ca^{2+}, and laminin (15 μg/ml) was added to the bottom well of a Transwell chamber (8 μM pore filters). The transfectants were resuspended in Puck's Saline A containing 0.5 mM Mn^{2+} and 0.1 mM Ca^{2+}, and 10^5 cells were added to the top well. After a 24-h incubation, the cells that had not migrated were removed, and the cells that had migrated onto the lower surface of the filters were fixed in methanol and then stained. Migration was quantitated by counting. The data shown are the mean values (±SEM) of three separate experiments done in duplicate. ■, control transfectants; ▨, PMA-stimulated transfectants.

and migration of the α6 transfectants. These results are important because they provide a functional rationale for the existence of multiple cytoplasmic domain variants of a specific integrin subunit.

The relative adhesive strengths of the α6A, α6B, and α6-ΔCYT transfectants correlate well with their ability to extend pseudopodia and to migrate toward a laminin gradient. This behavior is consistent with the recent work of DiMilla *et al.* (1993) that demonstrated that the ability of cells to migrate is a function of their initial attachment strength. More specifically, they observed a biphasic dependence of cell migration speed on substratum adhesive strength, i.e., maximal migration occurs at an intermediate attachment strength. The α6A transfectants exhibited a stronger adhesive strength for laminin than the α6B transfectants, and they migrated three- to fourfold better than the α6B transfectants. This result suggests that the α6A transfectants are closer to their optimal adhesive strength for migration than the α6B transfectants. This correlation is strengthened by the finding that deletion of the α6 cytoplasmic domain resulted in a decrease in both attachment strength and ability to migrate toward laminin in comparison to the α6A and α6B transfectants. Although PMA activation increased the adhesive strength of both the α6A and α6B transfectants for laminin, it caused a slight but reproducible decrease in the ability of these cells to migrate. Using the biphasic model, this observation could be explained by suggesting that PMA increases the attachment strength of the transfectants so that they exceed the intermediate strength that is optimal for migration. However, our data do not support this possibility because PMA activation increased the adhesive strengths of both the α6A and α6B transfectants for laminin to the same level, but the α6A transfectants still migrated three- to fourfold better than the α6B transfectants. This result suggests that the α6A and α6B transfectants may differ not only in adhesive strength but in other requirements for migration such as those associated with outside-in signaling.

The data obtained on the α6-ΔCYT transfectants in this study demonstrate that the α6 cytoplasmic domain plays a critical role in α6β1-mediated migration. The α6-ΔCYT transfectants were significantly impaired in their ability to migrate toward a laminin gradient even in the presence of Mn^{2+}, which promotes their attachment to laminin. Most likely, this behavior can be attributed to the emerging concept that migration on a matrix substratum requires a cascade of inside-out and outside-in signaling events that trigger cycles of integrin-mediated attachment and detachment (e.g., Regen and Horwitz, 1992; Dickinson and Tranquillo, 1993). Although the inside-out signaling requirement for the α6-ΔCYT transfectants can be obviated by Mn^{2+}, the α6 cytoplasmic domain is still necessary to transmit outside-in signals. Our observation that some migration of the α6-ΔCYT transfectants occurs in the presence of Mn^{2+} suggests that the β1 cytoplasmic domain is sufficient to support a basal level of migration but that the presence of an α6 cytoplasmic domain significantly enhances this migration. Additional evidence to support a critical role for the α6 cytoplasmic domain in signaling migration is provided by the laminin and divalent cation titration studies. PMA shifted both the divalent cation sensitivity and adhesive strength of the α6-ΔCYT transfectants to levels that were observed for the α6A transfectants under constitutive conditions, but it did not increase their ability to migrate (Figures 4 and 8). As discussed above, such data suggest that the α6 cytoplasmic domain also facilitates the transmission of outside-in signals required for migration.

The results presented in this paper should be compared to a recent study of the α6Aβ1 and α6Bβ1 receptors in K562 cells that concluded that the α6B transfectants adhered better to laminin than the α6A transfectants after PMA activation (Delwel *et al.*, 1993). The disparity between our data and those of Delwel *et al.* (1993) may be explained by the possibility that the cellular environments of K562 and P388D1 cells confer different properties on transfected integrins. This possibility is supported by several studies that have demonstrated a role for the cellular environment in regulating integrin function (reviewed in Hynes, 1992).

In addition to their differences in adhesive strength for laminin, the α6A and α6B transfectants differed in their divalent cation sensitivity for adhesion. All integrin α subunits contain three to five putative cation binding sites, and the activity of each αβ heterodimer can be modulated by divalent cations (Staatz *et al.*, 1989; Loftus *et al.*, 1990; Altieri, 1991; Kirchhofer *et al.*, 1991; Hynes, 1992). The ability of cations to bind directly to these sites has been demonstrated for several integrin receptors (Gailit and Ruoslahti, 1988; Smith and Cheresh, 1991, Michishita *et al.*, 1993). Two recent studies examined the functional significance of cation binding by mutating the divalent cation binding domains of the αM and α4 subunits (Masumoto and Hemler, 1993; Michishita *et al.*, 1993). These mutant receptors exhibited decreased cation sensitivity and diminished functional activity. Additional evidence to support the importance of divalent cations for integrin receptor activation has been the identification of antibodies that recognize divalent cation-dependent epitopes that are present only on activated receptors (Dransfield and Hogg, 1989; van Kooyk *et al.*, 1991). These findings suggest that divalent cations can influence receptor conformation and in so doing, contribute to activation of receptor function. In this study, we used divalent cations to examine the relative activation states of the α6 transfectants. The data we obtained indicate that the α6A and α6B cytoplasmic domains can differentially modulate the Ca^{2+}- and Mn^{2+}-binding properties of the α6A and α6B transfectants respectively. Moreover, the divalent cation data provide additional evidence to

support the conclusion drawn from the laminin titration data that specific sequences within the α6 cytoplasmic domains influence the activity of the extracellular domains.

An important question that arises from the data presented is how the α6 cytoplasmic domain sequences influence adhesive strength and cation binding activity. In the simplest of models, it can be postulated that the α6 cytoplasmic domain sequence confers a specific conformation on the extracellular domain. It would be premature, however, to conclude that the α6 cytoplasmic domains regulate the laminin-binding affinity of α6β1 in the absence of other molecules. This model, for example, would not account for the upregulation of receptor function observed in response to PMA stimulation. It could be argued that posttranslational modification of the α6 cytoplasmic domains alters receptor conformation. However, the only known modification of α6 is serine phosphorylation of the α6 cytoplasmic domain (Shaw *et al.*, 1990; Hogervorst *et al.*, 1993a), and we (Shaw and Mercurio, 1993) and others (Hogervorst *et al.*, 1993b) have recently demonstrated that the two serine residues in this domain are not essential for upregulation of receptor function. For these reasons, the possibility that specific cytoplasmic/cytoskeletal proteins interact preferentially with either the α6Aβ1 or α6Bβ1 cytoplasmic domains to facilitate or restrict receptor function should be considered.

It is interesting to compare the results obtained in this study with several other studies that have examined the relative expression of the α6A and α6B variants. Such studies have noted, for example, tissue specific expression of these variants (Hogervorst *et al.*, 1993a). Also, the differentiation of mouse embryonic stem cells, which involves alterations in cell migration, is associated with a change from α6B to α6A expression (Cooper *et al.*, 1991). In one recent study of particular interest, α6A and α6B expression was examined by immunohistochemistry in the developing chick retina (de Curtis and Reichardt, 1993). A spatial distribution of the two α6 variants was observed; the α6B subunit was expressed throughout the retina, whereas the α6A subunit was expressed only in a small region of the retina proximal to the optic nerve and on the optic nerve itself. This pattern of α6A expression may correlate with its requirement for migration of the optic nerve. Also, inflammatory macrophages, which are characterized by their ability to migrate toward specific stimuli, express only the α6Aβ1 variant (Shaw *et al.*, 1993). It appears from these observations that a correlation between α6A expression and motility may exist, and this correlation is supported by the mechanistic studies presented here.

Two other integrins with laminin receptor function, α3β1 and α7β1, have structural variants that differ only in their α subunit cytoplasmic domain sequences (Tamura *et al.*, 1991; Collo *et al.*, 1993). The α3, α6, and α7 integrins share a higher sequence homology to each other than to other integrin α subunits and, for this reason, it has been hypothesized that they arose from a common ancestral gene (Sastry and Horwitz, 1993). An obvious question that arises is whether the structural homology among these α subunits is reflected in functional similarities. Specifically, it will be interesting to determine whether the A and B structural variants of the α3 and α7 subunits differ in their adhesive strength and ability to mediate migration or other cellular behaviors given the results obtained with the α6 variants in this report.

This paper is dedicated to the memory of Eric Holtzman.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant CA-42276. L. Shaw is a Ryan Fellow at Harvard Medical School. A. Mercurio is the recipient of an American Cancer Society Faculty Research Award.

REFERENCES

Altieri, D.C. (1991). Occupancy of CD11b/CD18 (Mac-1) divalent cation binding site(s) induces leukocyte adhesion. J. Immunol. *147*, 1891–1898.

Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G., and Silengo, L. (1990). A human integrin β1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. Gene *95*, 261–266.

Bauer, J.S., Varner, J., Schreiner, C., Kornberg, L., Nicholas, R., and Juliano, R.L. (1993). Functional role of the cytoplasmic domain of the integrin α5 subunit. J. Cell Biol. *122*, 209–221.

Briesewitz, R., Kern, A., and Marcantonio, E.E. (1993). Ligand-dependent and independent integrin focal contact localization: the role of the α chain cytoplasmic domain. Mol. Biol. Cell *4*, 593–604.

Chan, B.M.C., Kassner, P.D., Schiro, J.A., Byers, H.R., Kupper, T.S., and Hemler, M.E. (1992). Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. Cell *68*, 1051–1060.

Clarke, A.S., and Mercurio, A.M. (1994). Evidence for a novel structural variant of the human β4 integrin cDNA. Cell Adhesion and Communication *2*, 1–6.

Collo, G., Starr, L., and Quaranta, V. (1993). A new isoform of the laminin receptor α7β1 is developmentally regulated in skeletal muscle. J. Biol. Chem. *268*, 19019–19024.

Cooper, H.M., Tamura, R.N., and Quaranta, V. (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the α6β1 integrin. J. Cell Biol. *115*, 843–850.

Cozen-Roberts, C., Lauffenberger, D.A., and Quinn, J.A. (1990). Receptor-mediated adhesion phenomenon: model studies with the radial-flow detachment assay. Biophys. J. *58*, 107–125.

de Curtis, I., and Reichardt, L.F. (1993). Function and spatial distribution in developing chick retina of the laminin receptor α6β1 and its isoforms. Development *118*, 377–388.

Delwel, G.O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R., and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin α6 subunit in transfected K562 cells. J. Biol. Chem. *268*, 26865–25875.

Dickinson, R.B., and Tranquilo, R.T. (1993). A stochastic model for cell random motility and haptotaxis based on adhesion receptor binding fluctuations. J. Math. Biol. 31, 563–600.

DiMilla, P.A., Stone, J.A., Quinn, J.A., Albeda, S.M., and Lauffenburger, D.A. (1993). Maximal migration of human smooth muscle cells on fibronectin and typeIV collagen occurs at an intermediate attachment strength. J. Cell Biol. 122, 729–737.

Dransfield, I., and Hogg, N. (1989). Regulated expression of Mg^{2+} binding epitope on leukocyte integrin α subunits. EMBO J. 8, 3759–3765.

Gailit, J., and Ruoslahti, E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. J. Biol. Chem. 263, 12927–12932.

Ginsberg, M.H., Du, X., and Plow, E.F. (1992). Inside-out integrin signaling. Curr. Opin. Cell Biol. 4, 766–771.

Grzesiak, J.J., Davis, G.E., Kirchhofer, D., and Pierschbacher, M.D. (1992). Regulation of α2β1-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Ca^{2+} and Mg^{2+}. J. Cell Biol. 117, 1109–1117.

Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990). Expression and function of chicken integrin b1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. 110, 175–184.

Hibbs, M.L., Xu, H., Stacker, S.A., and Springer, T.A. (1991). Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin β subunit. Science 251, 1611–1613.

Hogervorst, F., Admiraal, L.G., Niessen, C., Kuikman, I., Janssen, H., Daams, H., and Sonnenberg, A. (1993a). Biochemical characterization and tissue distribution of the A and B variants of the integrin α6 subunit. J. Cell Biol. 121, 179–191.

Hogervorst, F., Kuikman, I., Geurts van Kessel, A., and Sonnenberg, A. (1991). Molecular cloning of the human α6 subunit. Alternative splicing of α6 mRNA and chromosomal localization of the α6 and β4 genes. Eur. J. Biochem. 199, 425–433.

Hogervorst, F., Kuikman, I., Noteboom, E., and Sonnenberg, A. (1993b). The role of phosphorylation in activation of the α6Aβ1 laminin receptor. J. Biol. Chem. 268, 18427–18430.

Hogervorst, F., Kuikman, I., von dem Borne, A.E.G. Kr., and Sonnenberg, A. (1990). Cloning and sequence analysis of β4 cDNA: an integrin subunit that contains a unique 118kD cytoplasmic domain. EMBO J. 9, 765–770.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11–25.

Kassner, P.D., and Hemler, M.E. (1993). Interchangeable a chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a β1 integrin. J. Exp. Med. 178, 649–660.

Kawaguchi, S., and Hemler, M.E. (1993). Role of the α subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. J. Biol. Chem. 268, 16279–16285.

Kirchhofer, D., Grzesiak, J., and Pierschbacher, M.D. (1991). Calcium as a potential physiological regulator of integrin-mediated cell adhesion. J. Biol. Chem. 266, 4471–4477.

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K., and Martin, G.R. (1982). Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21, 6188–6193.

Languino, L.R., and Ruoslahti, E. (1992). An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. J. Biol. Chem. 267, 7116–7120.

Leavesley, D.I., Schwartz, M.A., Rosenfeld, M., and Cheresh, D.A. (1993). Integrin β1- and β3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. J. Cell Biol. 121, 163–170.

Loftus, J.C., O'Toole, T.E., Plow, E.F., Glass, A., Frelinger III, A.L., and Ginsberg, M.H. (1990). A β3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249, 915–918.

Marcantonio, E.E., Guan, J.-L., Trevithick, J.E., and Hynes, R.O. (1990). Mapping of the functional determinants of the integrin β1 cytoplasmic domain by site-directed mutagenesis. Cell Regul. 1, 597–604.

Masumoto, A., and Hemler, M.E. (1993). Mutation of putative divalent cation sites in the α4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. J. Cell Biol. 123, 245–253.

Mercurio, A.M., and Shaw, L.M. (1988). Macrophage interactions with laminin. PMA selectively induces the adherence and spreading of mouse macrophages on a laminin substratum. J. Cell Biol. 107, 1873–1880.

Michishita, M., Videm, V., and Arnaout, M.A. (1993). A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell 72, 857–867.

O'Toole, T.E., Mandelman, D., Forsyth, J., Shattil, S.J., Plow, E.F., and Ginsberg, M.H. (1991). Modulation of the affinity of integrin αIIbβ3 (GPIIB-IIIa) by the cytoplasmic domain of αIIb. Science 254, 845–847.

Regen, C.M., and Horwitz, A.F. (1992). Dynamics of β1 integrin-mediated adhesive contacts in motile fibroblasts. J. Cell Biol. 119, 1347–1359.

Reszka, A.A., Hayashi, Y., and Horwitz, A.F. (1992). Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. 117, 1321–1330.

Sastry, S., and Horwitz, A.F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling. Curr. Opin. Cell Biol. 5, 819–831.

Schwartz, M.A. (1993). Transmembrane signaling by integrins. Trends Cell Biol. 2, 304–308.

Shaw, L.S., Lotz, M., and Mercurio, A.M. (1993). Inside-out integrin signalling in macrophages. Analysis of the role of the α6Aβ1 and α6Bβ1 integrin variants in laminin adhesion by cDNA expression in an α6 integrin deficient macrophage cell line. J. Biol. Chem. 268, 11401–11408.

Shaw, L.M., and Mercurio, A.M. (1989). Interferon-gamma and lipopolysaccharide promote macrophage adherence to basement membrane glycoproteins. J. Exp. Med. 169, 303–308.

Shaw, L.M., and Mercurio, A.M. (1991). Regulation of α6β1 integrin function by divalent cations and protein phosphorylation. J. Cell Biol. 115, 131a (Abstract).

Shaw, L.M., and Mercurio, A.M. (1993). Regulation of α6β1 integrin laminin receptor function by the cytoplasmic domain of the α6 subunit. J. Cell Biol. 123, 1017–1025.

Shaw, L.M., Messier, J.M., and Mercurio, A.M. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the α6β1 integrin. J. Cell Biol. 110, 2167–2174.

Smith, J.W., and Cheresh, D.A. (1991). Labeling of integrin αvβ3 with ^{58}Co (III). J. Biol. Chem. 266, 11429–11432.

Solowska, J., Guan, J.-L., Marcantonio, E.E., Trevithick, J.E., Buck, C.A., and Hynes, R.O. (1989). Expression of normal and mutant avian integrin subunits in rodent cells. J. Cell Biol. 109, 853–861.

Sonnenberg, A., Modderman, P.W., and Hogervorst, F. (1988). Laminin receptor on platelets is the integrin VLA-6. Nature 336, 487–489.

Staatz, W.D., Rajpara, S.M., Wayner, E.A., Cater, W.G., and Santoro, S.A. (1989). The membrane glycoprotein la-IIa (VLA-2) complex mediates the Mg^{2+}-dependent adhesion of platelets to collagen. J. Cell Biol. *108*, 1917–1924.

Suzuki, S., and Naitoh, Y. (1990). Amino acid sequence of a novel integrin $\beta 4$ subunit and primary expression of the mRNA in epithelial cells. EMBO J. *9*, 757–763.

Tamura, R.N., Cooper, H.M., Collo, G., and Quaranta, V. (1991). Cell type-specific integrin variants with alternative α chain cytoplasmic domains. Proc. Natl. Acad. Sci. USA *88*, 10183–10187.

Tamura, R.N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L.F., Cooper, H.M., and Quaranta, V. (1990). Epithelial integrin $\alpha 6\beta 4$: complete primary structure of $\alpha 6$ and variant forms of $\beta 4$. J. Cell Biol. *111*, 1593–1604.

Trinkaus, J.P. (1984). Cells into Organs: The Forces That Shape the Embryo, 2nd ed., Englewood Cliffs, NJ: Prentice-Hall.

van Kooyk, Y., Weder, P., Hogervorst, F., Verhoeven, A.J., van Seventer, G., te Velde, A.A., Borst, J., Keizer, G.D., and Figdor, C.G. (1991). Activation of LFA-1 through a Ca++-dependent epitope stimulates lymphocyte adhesion. J. Cell Biol. *112*, 345–354.

van Kuppevelt, Toin H.M.S.M., Languino, L.R., Gailit, J.O., Suzuki, S., and Ruoslahti, E. (1989). An alternative cytoplasmic domain of the integrin $\beta 3$ subunit. Proc. Natl. Acad. Sci. USA *86*, 5415–5418.

Ylanne, J., Chen, Y., O'Toole, T.E., Loftus, J.C., Takada, Y., and Ginsberg, M.H. (1993). Distinct functions of integrin α and β subunit cytoplasmic domains in cell spreading and formation of focal adhesions. J. Cell Biol. *122*, 223–233.

Ziober, B.L., Vu, M.P., Waleh, N., Crawford, J., Lin, C.S., and Kramer, R.H. (1993). Alternative extracellular and cytoplasmic domains of the integrin $\alpha 7$ are differentially expressed during development. J. Biol. Chem. *268*, 26773–26783.

A new alternative transcript encodes a 60 kDa truncated form of integrin β_3

Isabelle DJAFFAR,*|| Yi-Ping CHEN,*§ Christophe CREMINON,† Jacques MACLOUF,* Anne-Marie CIEUTAT,* Odile GAYET‡ and Jean-Philippe ROSA*

*Unité 348 INSERM, Hôpital Lariboisière, 75475 Paris Cedex 10, France, †Service de Pharmacologie et d'Immunologie, DRIPP, CEA-Saclay, 91191 Gif-sur-Yvette, France, and ‡Unité 331 INSERM, Faculté de Médecine Alexis Carrel, 69372 Lyon Cedex 08, France

A cDNA for integrin β_3 isolated from a human erythroleukaemia (HEL) cell library contained a 340 bp insert at position 1281. This mRNA, termed β_{3C}, results from the use of a cryptic AG donor splice site in intron 8 of the β_3 gene, and is different from a previously described alternative β_3 mRNA. The predicted open reading frame of β_{3C} stops at a TAG stop codon 69 bp downstream from position 1281. It starts with the signal peptide and the 404 N-terminal extracellular residues of β_3, encompassing the ligand binding sites, followed by 23 C-terminal intron-derived residues, corresponding to a truncated form of β_3 lacking the cysteine-rich, transmembrane and cytoplasmic domains. Expression of β_{3C} mRNA was demonstrated in human platelets, megakaryocytes, endothelial cells and HEL cells by reverse transcriptase/PCR. The β_{3C} transcript was also demonstrated in the mouse, suggesting its conservation through evolution. Finally, a 60 kDa polypeptide corresponding to the β_{3C} alternative transcript was demonstrated in platelets by Western blotting using a polyclonal antibody raised against a synthetic peptide designed from the β_{3C} intronic sequence. Taken together, these results suggest a biological role for β_{3C}, the first alternative transcript showing an altered extracellular domain of a β integrin.

INTRODUCTION

The integrins are a superfamily of cell surface receptors that mediate cell–cell and cell–matrix adhesion [1–3]. They have become a subject of extensive study because of their profound biological importance in development, wound healing, metastasis, inflammation, immune responses, and thrombosis and haemostasis [1,2]. Eight subfamilies of integrins have been described to date. All members within each subfamily share a common β subunit, which is non-covalently associated with an α subunit. For example, in the β_3 subfamily, $\alpha_{IIb}\beta_3$ (also termed GPIIb–IIIa), the fibrinogen receptor on platelets, and $\alpha_v\beta_3$ (also termed VNR), a vitronectin receptor expressed by several cell types, share the same β_3 subunit. Some β subunits such as β_3 are more widely distributed than the associated α subunit (α_{IIb}), which is restricted to platelets and cells of megakaryocytic potential [4]. The β subunits of all integrins are remarkably similar in structure: a signal peptide at the N-terminus, a large extracellular domain containing ligand recognition sequence(s) and four cysteine-rich repeats, a transmembrane domain, and a short cytoplasmic tail at the C-terminus [1,5–18]. The amino acid sequences of all the β subunits are highly similar, with specific structural features being conserved over a wide variety of species [19].

$\alpha_{IIb}\beta_3$ mediates platelet aggregation via binding of adhesive proteins, mainly fibrinogen, fibronectin and von Willebrand factor [4]. Like other integrins, it provides a link between extracellular ligands and cytoskeletal components. The extracellular domain, particularly the N-terminal portion of the receptor, is involved in direct interactions with ligands. Residues 109–171 of β_3 are involved in the recognition of the tripeptide Arg-Gly-Asp [20,21], which is present in a number of matrix proteins and is of widespread importance in cell adhesion [2]. Residues 204–229 are also involved in fibrinogen binding [22]. Similarly, an α_{IIb} extracellular sequence is involved in the specific recognition of the C-terminal dodecapeptide of the fibrinogen γ chain [23]. Binding of soluble ligands to $\alpha_{IIb}\beta_3$ requires a conformational change of the receptor, which is probably induced by intracellular signalling, maybe via the cytoplasmic tail of β_3 [24]. Another identified function of the cytoplasmic tails of β integrins is incorporation into focal contact sites through interactions with cytoskeletal components, namely talin, vinculin and α-actinin for β_1 and β_3 [25], an essential step in the machinery linking the cytoskeleton and extracellular matrix.

Aside from the many combinations of α and β subunits, an additional mechanism that increases the diversity of the integrin superfamily is provided by alternative splicing. This has been described for both α and β subunits [26–30]. Interestingly, in the latter case, all alternative transcripts so far described have contained the β cytoplasmic tails. In the case of β_1, two alternative transcripts were found [28,29], one at least of which encoded a β_1 cytoplasmic variant that is functionally different from regular β_1, as indicated by its non-incorporation into adhesion plaques [30]. In the case of β_3 van Kuppevelt et al. [27] have reported an alternatively spliced mRNA which also generated an alternative cytoplasmic domain of β_3, but actual expression of a corresponding translation product was never demonstrated. We report here a new β_3 alternative transcript, the first β integrin alternative mRNA which truncates the extracellular domain and excludes the cytoplasmic domain. We analyse its tissue expression at the mRNA level and demonstrate expression of a corresponding protein product.

MATERIALS AND METHODS

Cloning and sequencing

A cDNA for β_{3C} was isolated from a human erythroleukaemia (HEL) cell cDNA library constructed in λgt10 [31]. It was subcloned either in M13mp18 phage vector or in pBluescript phagemid (Stratagene, San Diego, CA, U.S.A.) for sequencing of

Abbreviations used: HEL cells, human erythroleukaemia cells; RT/PCR, reverse transcription/PCR.

§ Present address: Committee on Vascular Biology CV-B2, Scripps Clinic Institute, La Jolla, CA 92037 U.S.A.

|| To whom correspondence should be addressed.

single-stranded DNA and double-stranded DNA respectively. Sequencing was performed by the dideoxy chain-termination method using Sequenase version 2.0 (USB, Cleveland, OH, U.S.A.); primers used were a universal M13 primer, T3 and T7 primers, or oligonucleotides derived from the β_3 cDNA sequence.

Preparation of cells and tissues

Human platelets were isolated as described [24]. Megakaryocytes were isolated from human femoral bone marrow using the magnetic beads method [26] (informed consent was obtained from patients undergoing surgery). Briefly, sheep anti-mouse IgG conjugated to magnetic beads (Dynal A.S., Compiègne, France) was incubated with monoclonal antibody P_2 (Immunotech, Marseille, France), specific for the $\alpha_{IIb}\beta_3$ complex, at room temperature for 30 min and then washed three times in RPMI-1640. Human bone marrow was aspirated through a 18-gauge needle and collected in RPMI-1640 containing 200 units/ml heparin (Sigma) and 5% fetal calf serum. The marrow suspensions were washed once in RPMI-1640 and then incubated with the P_2/sheep anti-mouse IgG/magnetic beads complex at room temperature for 30 min. Cells with surface-bound P_2–beads were purified using a magnetic concentrator (Dynal A.S.) according to the manufacturer's instructions. About 30% of the nucleated cells were megakaryocytes, as determined morphologically using Wright–Giemsa stains. HEL cells were obtained from the American Type Cell Culture Collection, and were cultured as described [32]. Lungs and kidneys from adult 3T3 mice were immediately frozen in liquid nitrogen, and kept at −80 °C until RNA extraction.

RNA isolation

Total cellular RNA was isolated from human platelets, megakaryocytes, smooth muscle cells and HEL cells as described [33], and from mouse lung and kidney using the guanidine isothiocyanate method followed by ultracentrifugation [34]. RNA from human umbilical endothelial cells was generously provided by M. Nakache, Hôpital Lariboisière, Paris, France.

Reverse transcription (RT)/PCR

An initial single-strand cDNA was synthesized from 1 μg (0.1 μg for megakaryocyte RNA) of human total cellular RNA with 200 units of Moloney murine leukaemia virus reverse transcriptase (BRL) and 1 μM primer E_1 (5′-CACAGATGCTCCAGGACAAA-3′; complementary to nucleotides 1322–1303 of β_{3C} mRNA, i.e. nucleotides 41–60 of intron 8 of the β_3 gene [35,36]). PCR was performed essentially as previously described [24] in a final volume of 100 μl containing 200 nM each of primers E_1 and F_3 (5′-AACTATAGTGAGCTCATCCC-3′; corresponding to nucleotides 1056–1075 of β_3 mRNA, and exon 7 of the gene), 200 μM of each dNTP, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.01% gelatin and 2.5 units of *Taq* DNA polymerase (Amersham-France, Les Ulis, France). After 5 min at 95 °C enzyme was added, and then PCR was performed for 30 temperature cycles (each of 94 °C/1.5 min, 55 °C/1.5 min and 72 °C/3 min steps; the last cycle included a 10 min/72 °C step) in an IHB thermal reactor (Hybaid Ltd., Teddington, Middlesex, U.K.). Genomic DNA PCR was performed under the same conditions, except that 1 mM $MgCl_2$ was used.

Reverse transcription and PCR amplification of β_{3C} mRNA from total RNA of mouse kidney and lung were performed as described above using primer E_1 (human intron 8) and primer F_{2S} (5′-CCCCACCACAGGCAATCAA-3′), which is derived from the sequence of mouse β_3 mRNA [34] and corresponds to nucleotides 599–617 (exon 3) of human β_3 mRNA. To prevent artefactual co-amplification of the human sequence, mouse primer F_{2S} was chosen because it exhibits four base differences from the corresponding human sequence. The annealing temperature in the PCR programme was elevated to 60 °C to prevent misannealing.

Production and characterization of anti-β_{3C} antibodies

To examine whether the β_{3C} mRNA was translated into an actual polypeptide, rabbit polyclonal antibodies were prepared against the synthetic peptide CPGASVGTGPPFFLL, corresponding to the C-terminal residues of the presumably translated intronic sequence of β_{3C}. The peptide was synthesized using a standard protocol with a Milligen 9050 apparatus (Waters). An immunogen was obtained by incubating 15 mg of the peptide and 15 mg of keyhole lympet haemocyanin overnight at 22 °C in the dark in the presence of 0.06% glutaraldehyde in 15 ml of phosphate buffer. No attempts were made to characterize the immunogen further. The conjugate (3 mg in 3 ml of saline) was emulsified with 3 ml of Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.), and 2 ml was injected subcutaneously into each of two rabbits. The first injection booster was given 6 weeks later (200 μg per animal), and rabbits were bled weekly. Boosters were performed every month and the same follow-up protocol was used. Antisera were tested (titre and sensitivity) in a competitive e.l.i.s.a. using the β_{3C} synthetic peptide covalently linked to acetylcholinesterase using the heterobifunctional reagent succinimidyl 4-[*NN*-maleimidomethyl)cyclohexane-1-carboxylate as tracer, as previously described [37]. The antiserum with optimal sensitivity was selected by its ability to displace the tracer with the β_{3C} peptide.

Western blot analysis

Platelet proteins (30 μg per lane) were separated by SDS/PAGE and blotted on to nitrocellulose according to standard procedures. Membranes were stained with Ponceau Red (5%) and then blocked overnight in 20 mM Tris, pH 7.6, 400 mM NaCl, 0.15% Tween-20 and 5% fat-free dry milk (Gloria, Courbevoie, France) at 4 °C. Incubations with primary antibodies were carried out overnight under mild agitation at 4 °C in the same buffer (TBS/Tween/milk). Where required, presaturation of the antibodies (serum diluted 1000-fold) by the synthetic peptide (100 μg/ml) was achieved in the same buffer at 4 °C overnight. Washes were for 4 × 10 min in TBS/Tween/milk and 2 × 20 min in TBS/Tween. Bound antibodies were detected by the ECL technique (Amersham-France) according to the manufacturer's instructions. Briefly, the secondary affinity-purified anti-rabbit IgG coupled to horseradish peroxidase was incubated in TBS/Tween/milk at a 1:5000 dilution for 30 min at room temperature. Washes were for 2 × 15 min and 2 × 5 min in TBS/Tween. Peroxidase activity was revealed by H_2O_2 and Luminol followed by film exposure (autoluminogram) for between 30 s and 15 min.

RESULTS

Nucleotide and corresponding amino acid sequence of β_{3C}

The nucleotide sequence of β_{3C} cDNA is identical to that of β_3 cDNA [7,31,38], with the exception of a 340 bp insertion at nucleotide position 1281 (Figure 1a). The open reading frame is

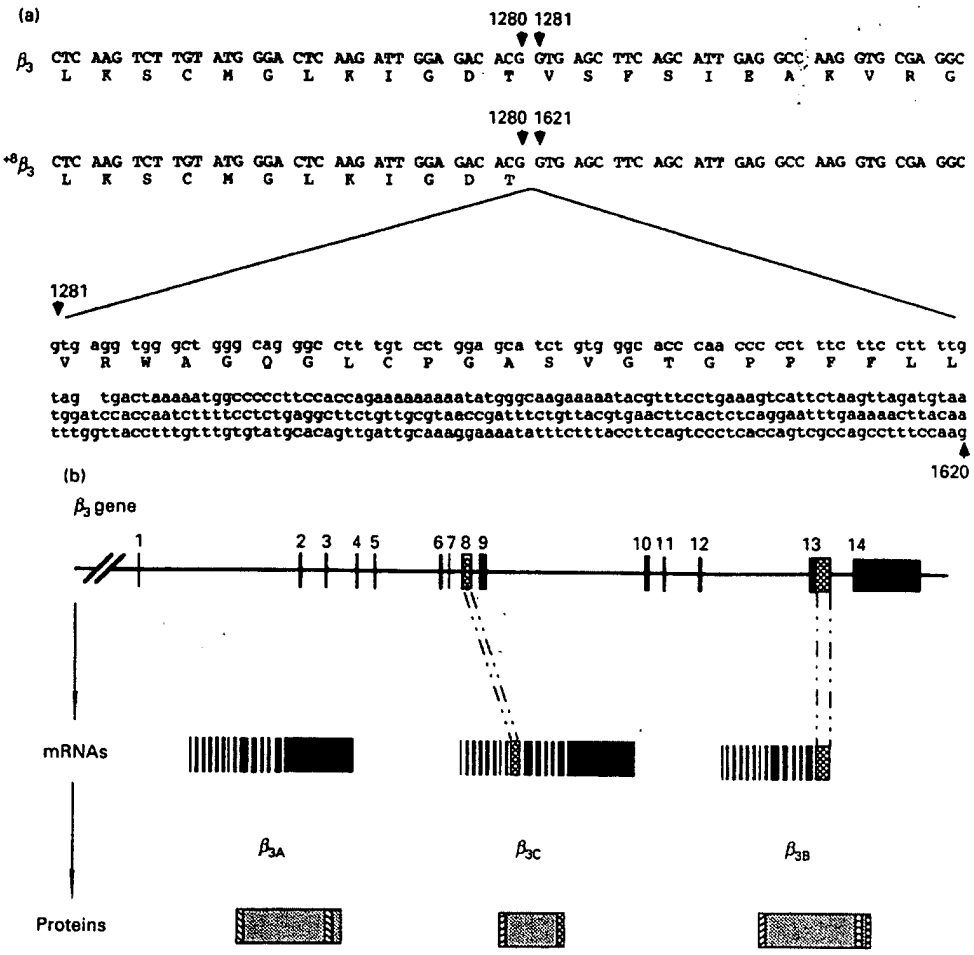


Figure 1 (a) Partial cDNA and deduced amino acid sequence of β_{3C}, and (b) splicing patterns of β_{3A}, β_{3C} and β_{3B}

(a) Nucleotide positions are indicated by numbers and arrows. Amino acids are indicated in the single-letter code. Part of the published β_3 sequence is shown for comparison [7,31,38]. The 340 bp internal sequence in β_{3C} indicated in lower case letters is not found in β_3. The open reading frame of β_{3C} extends within this sequence for 69 nucleotides before reaching a TAG stop codon. (b) ■, exons and corresponding sequences in mRNA; ▨, alternatively spliced-in parts of the introns; ▨, signal peptides and transmembrane domains. The splicing at the usual sites results in the longest open reading frame (788 amino acids) of β_3 with the transmembrane and cytoplasmic domains. The splicing in of the 5′ part of intron 8 results in an alternative form, β_{3C}, with a frame shift leading to premature termination. The intron 13-in and exon 14-out form of splicing previously reported [27] results in β_{3B}, with an alternative cytoplasmic domain.

not interrupted by the inserted sequence, but extends within the intervening sequence until a premature TAG stop codon, 69 nucleotides downstream from position 1280. Therefore β_{3C} cDNA encodes a putative protein composed of the 26-amino-acid signal peptide, the first 378 N-terminal amino acids of mature β_3, and an additional C-terminal 23 amino acids encoded by the inserted sequence. However, it does not contain the cysteine-rich repeats, or the transmembrane and cytoplasmic domains of β_3. Thus the putative β_{3C} polypeptide could correspond to a secretable subspecies of β_3. No sequence identity could be found between the C-terminal 23 amino acids of β_{3C} and sequences in the GenBank, NBRF and SwissProt data bases. Within this protein there are 14 cysteine residues, one of which lies within the C-terminal 23 intron-derived amino acids; the remaining 13 are in the upstream sequence. There are also three potential N-linked glycosylation sites.

Comparison of β_{3C} with β_3 and β_{3B}

Comparison with the genomic sequence of β_3 showed that the 340 bp insert of the β_{3C} cDNA corresponds to the first 340 bases of intron 8 of the β_3 gene [35] (Figure 1b). Hence this cDNA corresponds to a β_3 mRNA containing intron 8 which is alternatively spliced at a cryptic GT donor site, located 340 bp downstream from the regular donor site of β_3 at the exon 8/intron 8 boundary [35,36] (Figure 1b). The AG acceptor site at the intron 8/exon 9 junction of β_{3C} is the same as for the regular splicing of β_3 [36]. An additional nucleotide A at position 1388 in the non-coding portion of the β_{3C} mRNA was not found in the published sequence of the β_3 intron 8 [35]. Whether this represents a polymorphism remains to be investigated.

β_{3C} differs from a previously reported alternative transcript that we term β_{3B} [27]. The latter has the same sequence as β_3 prior to nucleotide position 2322, after which it diverges. In contrast to β_{3C}, β_{3B} contains neither the sequence from intron 8 nor the sequence from exon 14, but instead contains the 5′ part of intron 13 as a result of the non-splicing of intron 13, within which there is a AATAAA polyadenylation site. The putative protein encoded by β_{3B} would be a subspecies of β_{3A} with an alternative cytoplasmic domain, contrary to the β_{3C} subspecies

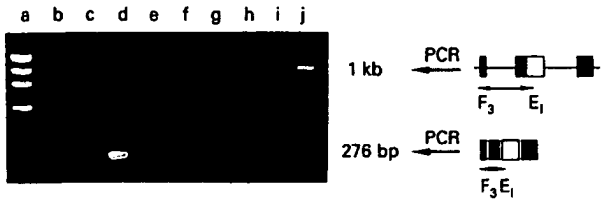


Figure 2 Detection of β_{3C} mRNA by the RT/PCR method

Total RNA prepared from human platelets, megakaryocytes, HEL cells and endothelial cells was reverse-transcribed. The resulting cDNAs were amplified by PCR. Primer E_I is derived from the spliced-in intronic sequence so as to amplify β_{3C} cDNA rather than β_3 cDNA. The PCR products were analysed by ethidium bromide staining of a 1.5% agarose gel. Lane a, ϕX174/*Hae*III-cut standard (Pharmacia, Uppsala, Sweden); lanes b, d, f and h are PCR amplifications of human platelets, megakaryocyte-enriched bone marrow cells, HEL cells and endothelial cells respectively; lanes c, e, g and i are control amplifications of the same cells, but without reverse transcriptase; lane j is the amplification of the genomic DNA from human peripheral leucocytes.

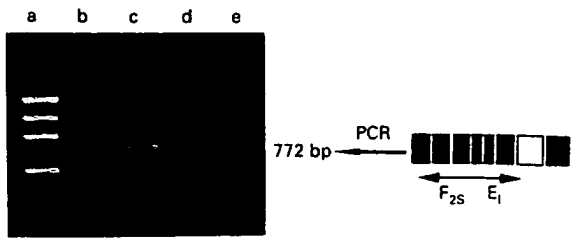


Figure 3 Detection of mouse β_{3C} mRNA by RT/PCR

Total RNA prepared from mouse kidney and lung was reverse-transcribed. The resulting cDNA was amplified by PCR. Primer E_I was described in the legend to Figure 2. Primer F_{2S} is derived from mouse β_3 cDNA [34]. The combination of the two primers specifically amplified cDNA from mouse β_{3C} mRNA. The PCR products were analysed by ethidium bromide staining of 1.5% agarose gel. Lane a, ϕX174/*Hae*III-cut standard; lanes c and e, amplifications of mouse kidney and lung respectively; lane b and d, control amplifications of the same tissues, but without reverse transcriptase. The drawing on the right represents relative positions of the primers relative to exons (■) and intron 8 in β_{3C} mRNA.

which does not contain the transmembrane and cytoplasmic domains (Figure 1b).

Detection of β_{3C} mRNA by PCR

We did not detect the β_{3C} transcript by the conventional Northern blotting method, either because of a low level of expression or because it is of a size too close to that of the regular transcript. We thus decided to use RT/PCR. An initial single-stranded cDNA for β_{3C} was reverse-transcribed from total cellular RNA using primer E_I, which is specific for intron 8 and therefore cannot prime regular β_{3A} mRNA. This cDNA was then amplified by PCR after addition of primer F_3, which was designed from exon 7. Amplification of β_3 transcripts could therefore be distinguished from amplification of potentially contaminating genomic β_3 sequence, since primer F_3 was separated from E_I by intron 7. β_{3C} mRNA was detected as a 276 bp PCR product in platelets (Figure 2, lane b), in megakaryocyte-enriched bone marrow cells (lane d), in HEL cells (lane f) and in endothelial cells (lane h). This 276 bp PCR product hybridized with a β_3 cDNA after Southern transfer, confirming its identity (results not shown). β_{3A} mRNA was expressed in all cells examined (results not shown). Thus β_{3C} RNA message is present in these cells and does not represent a cloning artefact from the HEL cDNA library.

Detection of mouse β_{3C} mRNA

To investigate whether β_{3C} mRNA is conserved in evolution, we amplified β_{3C} mRNA using the human intron 8 primer E_I and the mouse primer F_{2S} (corresponding to human exon 3) from the total RNA of mouse kidney and lung (Figure 3). A 772 bp product was obtained, indicating the presence of a mouse β_{3C} mRNA. Amplification was restricted to mouse β_{3C} mRNA, since primer F_{2S} was derived from the mouse β_3 sequence and contained four mismatches with the human β_3 mRNA [34]. Primer E_I, which is derived from the human intronic coding sequence of β_{3C} mRNA, was used for direct testing of the presence of related intronic mouse sequence. The size of the product obtained was identical to the predicted distance between the two primers on the human β_{3C} cDNA, and further suggests that mouse β_{3C} mRNA is very similar to its human counterpart.

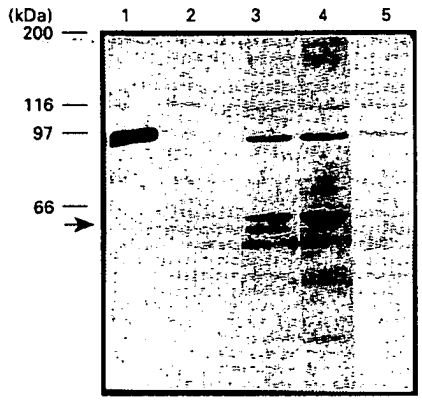


Figure 4 Detection of the β_{3C} polypeptide product by Western blotting

Platelet proteins (30 μg per lane) were separated by SDS/PAGE and blotted on to nitrocellulose membranes. Transferred proteins were reacted with specific antibodies, and bound IgG was detected with a secondary anti-rabbit IgG antibody coupled to horseradish peroxidase by the Luminol method and autoluminography. The exposure time was 30 s. Lane 1, polyclonal anti-β_{3A} (anti-GPIIIa) antiserum (1:20000 dilution); lane 2, non-immune serum (1:1000 dilution); lane 3, anti-(β_{3C} synthetic peptide) (1:1000 dilution); lane 4, anti-β_{3C} antibody (1:1000) preincubated with an excess of β_{3C} synthetic peptide (100 μg); lane 5, non-immune serum. The arrow on the left indicates the 60 kDa band detected by anti-(β_{3C} synthetic peptide) in lane 3, which disappears in the presence of excess free peptide in lane 4. Bands above and below correspond to background signal, since they are not sensitive to excess synthetic peptide.

Demonstration of a 60 kDa protein corresponding to the translation product of β_{3C}

Both its expression in normal tissues and its presence in at least one other species suggested biological significance for the β_{3C} transcript. We therefore decided to examine whether a protein product corresponding to β_{3C} was synthesized in normal tissues. Figure 4 shows a Western blot of whole platelet proteins treated with an anti-β_{3C} specific antibody raised against a synthetic peptide designed from the last 15 residues of the β_{3C} intronic sequence (lane 3) or the same antibody saturated with an excess of β_{3C} synthetic peptide (lane 4). A single band of 60 kDa was detected with the anti-β_{3C} antibody, which disappeared when the antibody was preincubated with an excess of β_{3C} synthetic peptide. This result demonstrated that the β_{3C} transcript was

translated into a polypeptide, further supporting its functional relevance.

DISCUSSION

We have identified a new alternative transcript for integrin β_3. Alternative mRNA splicing is a mechanism known for providing diversity in protein function and in the regulation of numerous genes [41]. It is therefore of potential interest to examine the exact nature of the alternative transcript of a given mRNA, its protein product and in some cases its regulation. The alternative β_{3C} mRNA which we identified in this study is due to the differential selection of a cryptic GT splice donor site 341 bp downstream from exon 8, resulting in the splicing in of 340 bp of the 5′ part of intron 8 of the β_3 gene. The corresponding AG acceptor splice site is the same as that for regular β_3 mRNA (β_{3A}) proposed by Lanza et al. [36], but is different from that proposed by Zimrin et al. [35]. In fact, the difference between the two propositions is a 5 bp shift for both the AG acceptor and GT donor sites, which does not affect the corresponding amino acids.

Alternative transcripts for integrin β subunits have been reported in the past, including two β_1 isoforms [28–30], one β_3 [27] and one β_4 [39] mRNA, leading to different cytoplasmic domains. All of these β integrin alternative mRNAs were generated by skipping of the last exon by non-splicing of the last intron or premature termination of transcription within the intron. This intron was in turn transcribed up to an alternative polyadenylation site. The mechanism described here for β_{3C} mRNA is very different, because there is no exon skipping or premature transcription termination, but partial splicing of intron 8 by use of a cryptic GT donor splice site, leaving the 5′ one-third of the intron unspliced. Moreover, the major difference in the present β_{3C} alternative sequence, in addition to the difference in terms of mechanisms, is that it is the first alternative transcript of a β integrin that differs in the extracellular region, and should lead to a truncated form of β_{3A} with no cytoplasmic or transmembrane domains.

The β_{3C} mRNA was detected by RNA PCR in platelets, megakaryocytes, endothelial cells and HEL cells. Because of the intronic nature of primer E_I, which restricted the amplification to the mRNA containing the corresponding sequence, the amplification was specific for β_{3C}. Attempts to detect the β_{3C} transcript by Northern blotting failed (results not shown). The simplest explanation is that β_{3C} is a minor transcript, as suggested by its weak PCR signal compared with β_3, and is therefore difficult to detect by the less sensitive Northern blotting method. Interestingly, a recent report using the B16a mouse cell line showed the presence of several β_3 bands (9, 7 and 6 kb) [40]. It is tempting to speculate that the 7 kb band represents β_{3C}, since mouse β_{3A} has a mobility close to 6 kb [32].

Intron-containing mRNA precursors that are slowly processed [41] can be easily detected by PCR. However, β_{3C} mRNA is not merely an unmatured precursor, since it is the product of the processing of intron 8 through use of a cryptic GT donor site, leaving only the 5′ third of the intron unspliced. In addition, the fact that an identical alternative transcript exists in mouse further indicates that β_{3C} is an actual transcript and not a partially processed β_3 mRNA precursor.

Our finding that β_{3C} alternative mRNA was also expressed in the mouse is highly significant, and further suggests that β_{3C} is biologically relevant. None of the several alternative β integrin transcripts previously reported in the literature were shown to be conserved in evolution. This emphasizes the potential importance of the β_{3C} transcript and prompted us to examine the possible expression of a corresponding protein product. Because of the splicing in of the 340 bp intronic sequence, the open reading frame of β_{3C} mRNA is shifted, and stops at a premature TAG stop codon 69 bp downstream from exon 8, thus encoding 23 new C-terminal amino acids. We used this predicted sequence to produce a synthetic peptide and raise an antibody specific for the putative β_{3C} polypeptide. We found that β_{3C} resulted in expression of a 60 kDa polypeptide in platelets. The size of this polypeptide is slightly higher than the 50 kDa predicted from the amino acid sequence, suggesting that it is glycosylated, in agreement with the three N-glycosylation sites present in the β_{3C} sequence.

Both the conservation of β_{3C} and, most importantly, its translation into a polypeptide, argue strongly in favour of its potential biological significance. The function(s) of this protein may be inferred from its structural features. (1) It lacks a transmembrane domain; since it contains the β_3 signal peptide, it could be either secreted constitutively or targeted to the α-granules as a secretory protein. Although β_{3C} encompasses residues 110–350, which seem to be involved in the association with α_{IIb} [42], the substantial structural differences from β_3 and the absence of the cysteine-rich domain render this association with α_{IIb} questionable. (2) β_{3C} lacks the cytoplasmic domain and therefore cannot interact with cytoskeletal proteins; as a consequence, even if it associated with α_{IIb} or α_v, it would probably not be incorporated into focal adhesions, a phenomenon independent from integrin α subunits [25]. (3) It must lack the tight structure of β integrins conferred by the cysteine-rich domain that is absent from β_{3C}. Though no particular function has been attached to the integrin cysteine-rich domain, it is likely to play a fundamental structural role, since it is conserved throughout evolution and across the whole superfamily [1]. (4) β_{3C} may bind RGD-containing adhesive ligands, since it encompasses the ligand-binding domains of β_3 represented by residues 91–171 and 211–222 [20–22].

β_{3C} shows considerable overall structural differences from β_{3A} which probably result in distinct ligand-binding characteristics. In β_{3C} the capacity to bind fibrinogen should be preserved, but its ability to be activated through conformational change may be lost. Speculation on a potential role for this β_{3C} polypeptide is tempting; for example, secreted β_{3C} could act as a competitive inhibitor of fibrinogen (or other adhesive ligand) binding to $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$, and thus contribute to the regulation of platelet aggregation, or of adhesion to the extracellular matrix, or else to endothelial cell migration. Experiments are now under way to test this hypothesis.

We thank P. Pradelles and J. Grassi for their helpful discussions during this work, and Marc Plaisance for his help in preparing the peptide immunogen. This project was supported in part by a grant from Institut des Vaisseaux et du Sang, and the Association Française des Myopathes. I.D. is the recipient of a fellowship from La Fondation pour la Recherche Médicale. Y.-P.C. is the recipient of a fellowship from La Foundation de France, and A.-M.C. of a fellowship from La Ligue Nationale Française Contre le Cancer.

REFERENCES

1 Hynes, R. O. (1987) Cell **48**, 549–554
2 Ruoslahti, E. and Pierschbacher, M. D. (1987) Science **238**, 491–497
3 Ruoslahti, E. (1991) J. Clin. Invest. **87**, 1–5
4 Phillips, D. R., Charo, I. F. and Scarborough, R. M. (1991) Cell **65**, 359–362
5 Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D. and Ruoslahti, E. (1987) J. Cell Biol. **105**, 1183–1190
6 Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. and Springer, T. A. (1987) Cell **48**, 683–690
7 Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Lo, S. and Phillips, D. R. (1987) J. Biol. Chem. **262**, 3936–3939
8 Kajiji, S., Tamura, R. M. and Quaranta, V. (1989) EMBO J. **8**, 673–680
9 Suzuki, S. and Naitoh, Y. (1990) EMBO J. **9**, 757–763

10 Hogervorst, F., Kuikman, L., von dem Borne, A. E. G. K. and Sonnenberg, A. (1990) EMBO J. **9**, 765–770
11 Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M. and Quaranta, V. (1990) J. Cell Biol. **111**, 1593–1604
12 McLean, J. W., Vestal, D. J., Cheresh; D. A. and Bodary, S. C. (1990) J. Biol. Chem. **265**, 17126–17131
13 Ramaswamy, H. and Hemler, M. E. (1990) EMBO J. **9**, 1561–1568
14 Suzuki, S., Huang, Z.-S. and Tanihara, H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5354–5358
15 Sheppard, D., Rozzo, C., Starr, L., Quaranta, V., Erle, D. J. and Pytela, R. (1990) J. Biol. Chem. **265**, 11502–11507
16 Yuan, Q., Jiang, W.-M., Krissansen, G. W. and Watson, J. D. (1990) Int. Immunol. **2**, 1097–1108
17 Erle, D. J., Rüegg, C., Sheppard, D. and Pytela, R. (1991) J. Biol. Chem. **266**, 11009–11016
18 Moyle, M., Napier, M. A. and McLean, W. (1991) J. Biol. Chem. **266**, 19650–19658
19 Albelda, S. M. and Buck, C. A. (1990) FASEB J. **4**, 2868–2880
20 D'Souza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C.-T. and Plow, E. F. (1988) Science **242**, 91–93
21 Smith, J. W. and Cheresh, D. A. (1988) J. Biol. Chem. **263**, 18726–18731
22 Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A. and Scarborough, R. M. (1991) J. Biol. Chem. **266**, 1415–1421
23 D'Souza, S. E., Ginsberg, M. H., Matsueda, G. P. and Plow, E. F. (1991) Nature (London) **350**, 66–68
24 Chen, Y.-P., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A.-M., Caen, J. P. and Rosa, J.-P. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10169–10173
25 Otey, C. A., Pavalko, F. M. and Burrdige, K. (1989) J. Cell Biol. **111**, 721–729
26 Bray, P. F., Leung, C. S.-I. and Shuman, M. A. (1990) J. Biol. Chem. **265**, 9587–9590
27 van Kuppevelt, T. H. S. M., Languino, L. R., Gailit, J. O., Suzuki, S. and Ruoslahti, E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5415–5418
28 Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G. and Silengo, L. (1990) Gene **95**, 261–266
29 Languino, L. R. and Ruoslahti, E. (1992) J. Biol. Chem. **267**, 7116–7120
30 Balzac, F., Belkin, A. M., Koteliansky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L. and Tarone, G. (1993) J. Cell Biol. **121**, 171–178
31 Rosa, J.-P., Bray, P. F., Gayet, O., Johnston, G. I., Cook, R. G., Jackson, K. W., Shuman, M. A. and McEver, R.-P. (1988) Blood **72**, 593–600
32 Rosa, J.-P. and McEver, R. P. (1989) J. Biol. Chem. **264**, 12596–12603
33 Djaffar, I., Vilette, D., Bray, P. F. and Rosa, J.-P. (1991) Thromb. Res. **62**, 127–135
34 Cieutat, A.-M., Rosa, J.-P., Letourneur, F., Poncz, M. and Rifat, S. (1993) Biochem. Biophys. Res. Commun. **193**, 771–778
35 Zimrin, A. B., Gidwitz, S., Lord, S., Schwartz, E., Bennett, J. S., White, G. C., II and Poncz, M. (1990) J. Biol. Chem. **265**, 8590–8595
36 Lanza, F., Kieffer, N., Phillips, D. R. and Fitzgerald, L. A. (1990) J. Biol. Chem. **265**, 18098–18103
37 McLaughlin, L., Wei, Y., Stockmann, P. T., Leahy, K. M., Needleman, P., Grassi, J. and Pradelles, P. (1987) Biochem. Biophys. Res. Commun. **144**, 469–476
38 Zimrin, A. B., Elsman, R., Vilaire, G., Schwartz, E., Bennett, J. S. and Poncz, M. (1988) J. Clin. Invest. **81**, 1470–1475
39 Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichart, L. F., Cooper, H. M. and Quaranta, V. (1990) J. Cell Biol. **111**, 1593–1604
40 Chen, Y. Q., Gao, X., Timar, J., Tang, D., Grossi, I. M., Chelladurai, M., Kunicki, T. J., Fligiel, S. E. G., Taylor, J. D. and Honn, K. V. (1992) J. Biol. Chem. **267**, 17314–17320
41 McKeown, M. (1992) Annu. Rev. Cell Biol. **8**, 135–155
42 Calvete, J. J., Mann, K., Alvarez, M. V., Lopez, M. M. and Gonzalez-Rodriguez, J. (1992) Biochem. J. **282**, 523–532

Received 1 October 1993; accepted 23 November 1993

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1993 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 268, No. 25, Issue of September 5, pp. 19019-19024, 1993
Printed in U.S.A.

A New Isoform of the Laminin Receptor Integrin α7β1 Is Developmentally Regulated in Skeletal Muscle*

(Received for publication, February 16, 1993, and in revised form, April 16, 1993)

Ginetta Collo, Lisa Starr, and Vito Quaranta‡

From the Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Within the integrin family, there are two groups of receptors that bind laminin. One of these groups comprises the heterodimers α3β1, α6β1, and α7β1, all of which bind the E8 fragment of laminin, and whose α subunits show significant homology at the amino acid sequence level. α3 and α6 exist as isoforms with distinct cytoplasmic domains (termed A and B), suggesting that they may couple laminin adhesion to distinct cellular responses. We report the identification of a new α7 mRNA which encodes an α7 protein isoform with an alternative cytoplasmic domain. Based on homology with α3 and α6 isoforms, this new isoform is classified as α7A and the previously published one as α7B. This result extends the similarity between α3, α6, and α7 laminin receptor subunits and suggests a common ancestral gene.

The α7β1 laminin receptor was proposed to be involved in myogenic differentiation. However, α7 isoforms were not investigated in that context. We detected the α7B isoform mRNA in all tissues and cell types tested, including myocardial and skeletal muscle. In contrast, the α7A isoform was detectable exclusively in skeletal muscle, not in myocardial muscle or cells or any other tissues or cell lines tested. Furthermore, the differentiating skeletal muscle cell line C2C12 expressed only α7B at the replicating myoblast stage and acquired α7A expression upon induction of differentiation and fusion. Splicing of α7B mRNA in C2C12 occurred shortly after myogenin expression and could be an indicator of progression through the program of skeletal muscle differentiation.

Cell-cell and cell-extracellular matrix interaction are important in the control of many biological events such as cell proliferation, differentiation, and migration (1-3). These events are mediated in part by integrins (4). Integrins are heterodimeric cell surface receptors composed of two noncovalently associated transmembrane subunits, α and β (5). At present, 8 β and 14 α subunits have been identified. The association of α and β subunits generate about 20 different receptors (6). The integrin extracellular domain forms a ligand-binding site recognizing one or more extracellular ligands or counter-receptors on other cells (6), and the cytoplasmic domain is known to interact with cytoskeletal proteins. In several subunits, alternatively spliced cytoplasmic domains have been identified, including β1 (7, 8), β3 (9), β4 (10, 11), α3 (12), and α6 (12, 13).

The integrin α7 subunit has been cloned recently and sequenced (14). It was originally identified by immunofluorescence in skeletal muscle and cardiac muscle cells as a developmentally regulated cell surface antigen (15). It is expressed in replicating myoblasts and expression increases during terminal differentiation. It is regulated by distinct mechanisms during muscle development (14, 16, 17) and is not detectable in developmentally defective myoblasts (14) or under conditions that inhibit myoblast differentiation (17). Murine myoblasts use the α7 subunit in association with β1 to bind laminin (18). In fact, laminin, but not collagen or fibronectin, has been shown to be important to maintain myoblast proliferation *in vitro* (19, 20).

The integrin α7 subunit has significant homology with the α3 and α6 subunits (14), and like α3 and α6 it associates with the β1 subunit (18), forming a subgroup within the integrin family (6). α7β1, like α3β1 and α6β1, binds to the E8 fragment of laminin (18, 21). On the basis of these similarities, we postulated the existence of a structural variant of α7 containing a distinct cytoplasmic domain as has been shown for α6 and α3 (12).

In the present study we report the identification of a new α7 isoform with an alternative cytoplasmic domain that we classify as α7A based on homology with the α3 and α6 isoforms. This result extends the similarities among α3β1, α6β1, and α7β1 laminin receptors and suggests that they are descendants of a common ancestral gene. The new α7 isoform is detectable exclusively in skeletal muscle. It is absent in cardiac muscle and in other tissues or cell lines tested. Its expression is concurrent with myoblast differentiation. Correlation of differential isoform expression with changes in cell morphology during skeletal myogenesis suggests a role for α7 in development, perhaps by coupling specific cellular responses to laminin through mediation of distinct interactions with cytoplasmic components.

EXPERIMENTAL PROCEDURES

Mouse Tissues—Tissues were dissected from 6-month-old female CB6/F1 mice, outbred from C57Black6 and BALB/c (Jackson Laboratories, Bar Harbor, ME). Tissue specimens were snap-frozen in liquid nitrogen and stored at −70 °C. For skeletal muscle samples, *upper back, abdominal, or upper leg muscle specimens were used* interchangeably with similar results.

Cell Lines—All murine cell lines were cultured in Dulbecco's modified Eagle's medium, 10% FCS,[1] 2 mM glutamine, and penicillin/

* This work was supported by National Institutes of Health Grants CA47858 and GM46902. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with *18* U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L16544.

‡ To whom correspondence should be addressed: The Scripps Research Inst., 10666 North Torrey Pines Rd., NX-7, La Jolla, CA 92037. Tel.: 619-554-9871, Fax: 619-554-6251.

[1] The abbreviations used are: FCS, fetal calf serum; TBE, Tris borate/EDTA electrophoresis buffer; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s).

streptomycin (50 IU/ml, 50 mg/ml), except B16F1 melanoma cells (5% FCS), and F9 mouse teratocarcinoma cells (plated on dishes precoated with 0.1% gelatin). C2C12 myoblasts were a gift from Dr. C. Glass (The Scripps Research Institute, La Jolla, CA) and were cultured in Dulbecco's modified Eagle's medium with 20% FCS to maintain replication or with 4% horse serum to induce differentiation (22). Primary cultures of rat myocardial cells were a gift from Dr. S. Evans (The University of California, San Diego, CA).

mRNA Amplification, Cloning, and Sequencing—Poly(A$^+$) RNA was isolated from cultured mouse cell lines or normal mouse tissue homogenates using the Micro-Fast Track mRNA Isolation Kit (Invitrogen, La Jolla, CA). About one million cells or 200 mg of tissue were used for each preparation. For mRNA extraction, samples were pulverized in a mortar with liquid nitrogen to a very fine powder and then transferred to a RNase-free microcentrifuge tube, resuspended in 1 ml of lysis buffer and processed according to the kit instructions. This kit should typically yield 1–5 μg of mRNA/100 mg of tissue. Single-stranded cDNA was synthesized from mRNA using the cDNA Cycle Kit (Invitrogen). Oligonucleotide primers flanking the predicted region of variation of the rat integrin α7 coding region were designed and synthesized (3118: 5′-CTACAGCTTTGACCGTGC-3′ and 3119: 5′-GAAAGGGTGGAAGGAACC-3′). PCR assays were carried out as described (12, 23), and the final PCR products were analyzed on 2% agarose/TBE buffer gels. Cloning of PCR products was carried out as described (24). Clones with correct size inserts were sequenced manually with the Sequenase Kit (United States Biochemical Corp.). Oligonucleotide primers 3154 (5′-GTTGTGGAAGGAGTCCC-3′) and 3155 (5′-GTCTTCCCGAGGGATCTT-3′) were synthesized from the resulting mouse α7 sequence. Myogenin oligonucleotide primers MyoGS (AGCTCCCTCACCAGGAGGA) and MyoGAS1 (GGGCTCTCTGGACTCCATCT) were a gift from Dr. Wanda Miller-Hance (The University of California, San Diego, CA). Yeast RNA (extracted from *Schizosaccharomyces pombe*) was a gift from Dr. Clare McGowan (The Scripps Research Institute, La Jolla, CA), and *Pyp3* yeast cDNA primers (25) were a gift from Dr. Jonathan B. A. Miller (The Scripps Research Institute, La Jolla, CA).

RESULTS

Identification of a New α7 Isoform with an Alternative Cytoplasmic Domain—The α3 and α6 integrin subunits have both been shown to have two alternatively spliced cytoplasmic domains, classified as A and B isoforms. The A isoform mRNAs contain a small insertion which is identically positioned within the B sequences of α3 and α6. Computer alignment of α7 with the α3 and α6 splice variants indicated that the published sequence of α7 was most likely a B isoform of the molecule. Because of known structural and functional similarities between the three subunits, we postulated the existence of an additional isoform of α7 corresponding to α3A and α6A. This variant would probably be spliced at the same junction as the α3A and α6A and contain an insertion of similar size (about 130 bp).

Amplification by reverse transcription-polymerase chain reaction (RT-PCR) (23) was carried out on mRNA extracted from mouse heart, brain, and skeletal muscle with oligonucleotide primers flanking the predicted region of variation of the rat α7 coding region (3118 and 3119). With these primers we expected a PCR product of 567 base pairs corresponding to the mouse version of the published rat α7 and another slightly larger product which should correspond to the predicted A isoform sequence. We obtained the one expected product of 567 bp from heart, brain, and muscle. However, skeletal muscle contained an additional product of 680 bp (Fig. 1). The two PCR fragments from muscle were subcloned and sequenced.

The nucleotide sequence of the smaller fragment was 93.7% identical to rat α7, indicating that it represented the murine homologue of the α7 subunit. The sequence of the larger product matched that of the smaller species except for a 113-bp internal insert located at precisely the same junction as the α3A and the α6A isoform splice sites. This insert was

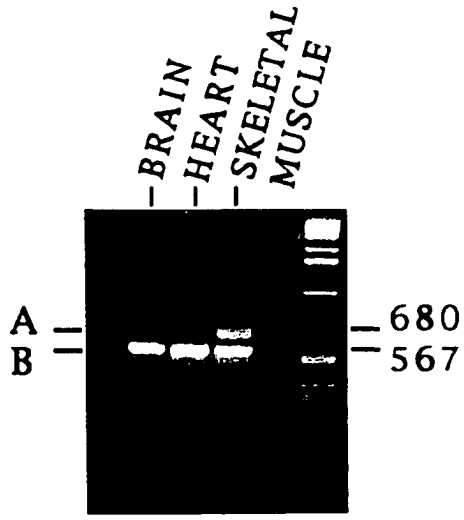


FIG. 1. **Identification of murine α7A and α7B mRNA isoforms by RT-PCR.** RT-PCR amplification performed on mouse brain, heart, and skeletal muscle mRNA with primers 3118 and 3119 (see text) resulted in a product consistent in size (567 bp) with the expected α7B isoform and an additional product of 680 bp in skeletal muscle, which was consistent with the predicted size of an α7A isoform.

similar in size to the α3A and the α6A inserts (144 and 130 bp, respectively). It modifies the α7 reading frame to encode a protein with an alternative cytoplasmic domain that is 19 amino acids shorter, and which contains the amino acid sequence GFFRR, a motif highly conserved among all integrin α chains (Fig. 2). Alignment of this alternative cytoplasmic sequence with the α3A and α6A tails (Fig. 3*a*) shows 47.2 and 55.6% respective identity at the protein level, indicating that the new isoform should be classified as α7A and the previously published sequence as α7B. Interestingly, in contrast to α3 and α6, the α7A tail coding region terminates beyond the inserted sequence, to form a 19-codon out-of-frame overlap with the B isoform tail coding region (see Fig. 2).

The α3, α6, α7 alignment suggests potentially important conserved motifs between and within the A and B tails (Fig. 3*a*). A basic residue two positions downstream from GFFKR is found in all A and B tails except for α7A. The invariant pair YH is present in all A and B tails, except for α3A. Following YH, there is a group of nine residues highly conserved within the A isoforms which can be reduced to a consensus sequence of KAE*XXX*QPS. Within the B tails, this region is also highly conserved, but with a distinct consensus represented by AVRIR*X*EER. The high degree of conservation suggests that this "nonamer box" may be of functional value. The fact that the nonamer consensus motif of the A forms is distinct from that of the B forms suggests also that it may be a site of functional divergence for the A and B isoforms.

Overall, the α3 and α6 isoform tails are more similar to each other than they are to α7: α3A and α6A are 62.9% identical, whereas the α7A tail is 47.2 and 55.6% identical to the α3A and α6A tails, respectively. The α3B and α6B tails are 66% identical, whereas the α7B tail is 52.9 and 56.6% identical, respectively, to α3B and α6B (numerical values were calculated from pairwise alignment of the cytoplasmic tails from GFFKR to termination with the University of Wisconsin GCG sequence analysis program "Bestfit"). Furthermore, the lengths of the isoforms of α3 and α6 are remarkably similar, whereas the α7 tails are both longer. The higher degree of homology between α6 and α3 suggests closer

```
GCCGTGCTTCACGTCTGGGGCCGCCTCTGGAACAGCACCTTTCTGGAGGAGTACATGGCCGTGAAATCCCTGGAAGTGATCGTCCGAGCCAACATCACAGTGAAATCCTCCATTAAGAACTTG
A V L H V W G R L W N S T F L E E Y M A V K S L E V I V R A N I T V K S S I K N L

TTGCTCAGAGATGCATCCACAGTGATTCCAGTGATGGTGTACTTGGACCCCATGGCCGTGGTTGTGGAAGGAGTCCCCTGGTGGGTCATCCTCCTGGCAGTGCTGGCTGGGCTGTTAGTCCTG
L L R D A S T V I P V M V Y L D P M A V V V E G V P W W V I L L A V L A G L L V L

GCCTTGCTGGTACTGCTGCTGTGGAAGgtggcttcttccgtcggaacagtccaagctcctcatttccaaccaactatcaccgagcccatctggccgtgcagccctcggccatggaggctggg
A L L V L L L W K C G F F R R N S P S S S S F P T N Y H R A H L A V Q P S A M E A G

ggtccagggactgtgggCTGGGATTCTTCAAGCGGGCGAAGCACCCCGAGGCCACCGTGCCCCAGTACCACGCAGTGAAGATCCCTCGGGAAGACCGACAGCAGTTCAAGGAGGAGAAGACAG
G P G T V G W D S S S G R S T P R P P C P S T T Q *

L G F F K R A K H P E A T V P Q Y H A V K I P R E D R Q Q F K E E K T G

GCACCATCCAGAGGAGTAACTGGGGCAACTCCCAGTGGGAGGGCTCCGACGCACACCCCATCTTGGCTGCCGACTGGCATCCTGAGCTGGGTCCTGATGGACATCCGGTGCCAGCCACTGCCT
T I Q R S N W G N S Q W E G S D A H P I L A A D W H P E L G P D G H P V P A T A *
```

FIG. 2. **Mouse α7A and α7B partial cDNA sequences.** Nucleic acid sequence and predicted protein translation of a fragment of mouse α7 cDNA encompassing the alternative spliced form of the cytoplasmic region. The A isoform insertion is *boxed*, and the resulting protein sequence is *shaded*. These sequences are deposited with the GenBank Accession Number L16544.

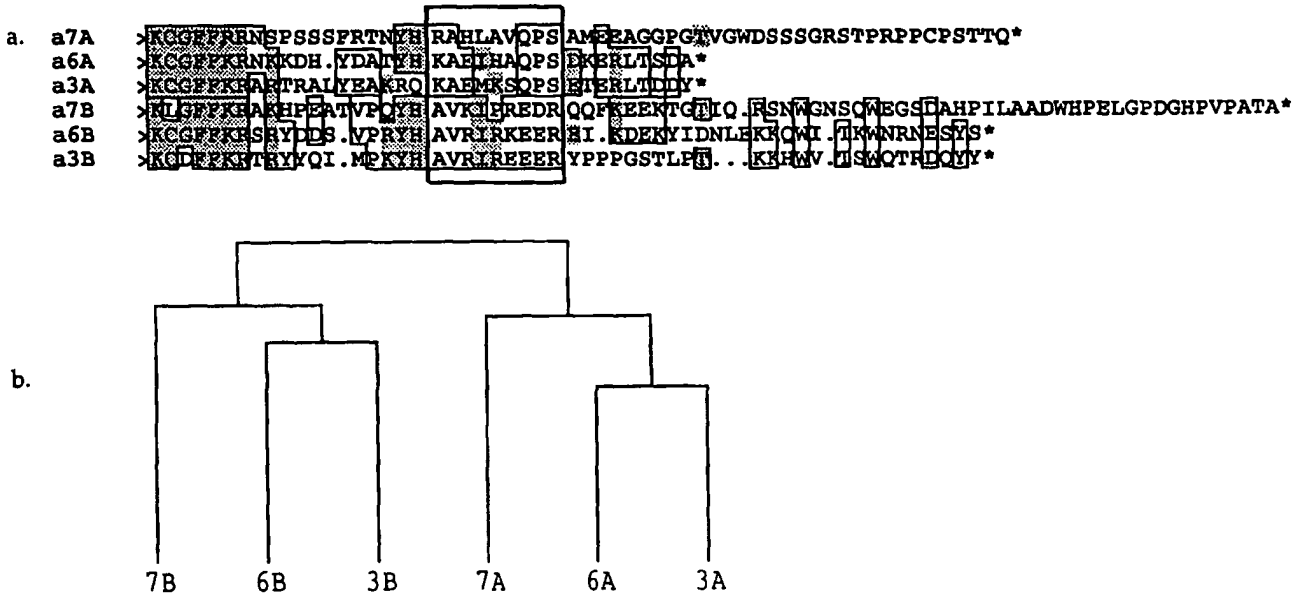


FIG. 3. *A*, alignment of predicted protein sequences of cytoplasmic regions of human α3, human α6, and mouse α7 A and B isoforms. Gaps were introduced to maximize homology. Identities at three or more positions between isoform sequences are *shaded* and those at two or more positions within isoform groups are *boxed*. For this alignment, lysine and arginine are considered equivalent, as are glutamic and aspartic acids. The *double box* encompasses a region of strong homology unique to each isoform group but identical in position and size, termed nonamer box (see text). *B*, predicted evolutionary relationship between α3, α6, and α7 A and B alternative cytoplasmic tails. Dendrogram was generated with Pileup (University of Wisconsin GCG sequence analysis programs).

evolutionary distance, as shown in the dendrogram in Fig. 3*b*, and may be an indication of less divergent functions.

Expression of α7 mRNA Isoforms in Mouse Tissues and Cell Lines—To investigate distribution of the two α7 isoforms, RT-PCR was carried out on mRNA extracted from additional mouse tissues with primers (3154 and 3155) derived from the mouse α7 sequence (Fig. 2). We expected a product of 283 bp corresponding to α7A mRNA and a product of 170 bp corresponding to α7B mRNA. In addition to the two expected bands, we occasionally found a third larger species, but only in samples containing the A isoform (Fig. 4). This third band proved to be a heteroduplex resulting from annealing of one strand each of A and B isoform DNA, as already observed for α3 and α6 (12). This was demonstrated by using as PCR templates either α7A or α7B cloned cDNAs or a mixture of the two. Products amplified from the A cDNA resolved as a single 283-bp band, products from the B cDNA resolved as a single 170-bp band, whereas the mixture of the two cDNAs yielded three products of 350 (approximately), 283, and 170 bp (not shown). The band at approximately 350 bp must, therefore, be a heteroduplex formed by the annealing of an A strand with a complementary B strand along their region of identity (Fig. 2). The abnormal migration on gel is probably due to a nonannealed loop corresponding to the extra sequences in the A strand.

Unexpectedly, all organs tested (stomach, intestine, liver, spleen, heart, skeletal muscle, kidney, brain, and ovary) contained α7B mRNA (Fig. 5*A*). We also tested the following mouse cell lines: 3T3 (fibroblasts), STO (immortalized mouse embryonic fibroblasts), MMT (mouse mammary carcinoma), B16 (melanoma), and F9 and P19 (teratocarcinomas). All of these cell lines contained α7 mRNA (B isoform) (Fig. 5). These results are in apparent conflict with previously published data (14, 18, 21), indicating a restricted distribution of α7. As shown in Fig. 5*B*, we included the following controls in our experiments.

To eliminate the possibility that primer preparations were either contaminated with α7 contaminating plasmids, or

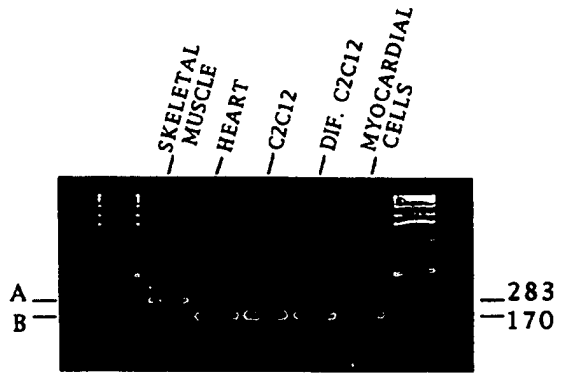


FIG. 4. **Expression of α7A and α7B mRNA in muscle cells or tissues.** RT-PCR was performed on mRNA extracted from mouse skeletal muscle, mouse heart, C2C12 replicating myoblasts, differentiated C2C12 forming myotubes, and a primary culture of rat myocardial cells using oligonucleotide primers (3154/3155) from the mouse α7 sequence. The 170-bp product corresponding to the α7B isoform is found in all samples tested. The product of 283 bp corresponds to the α7A isoform and appears to be exclusively expressed in skeletal muscle and differentiated myoblasts.

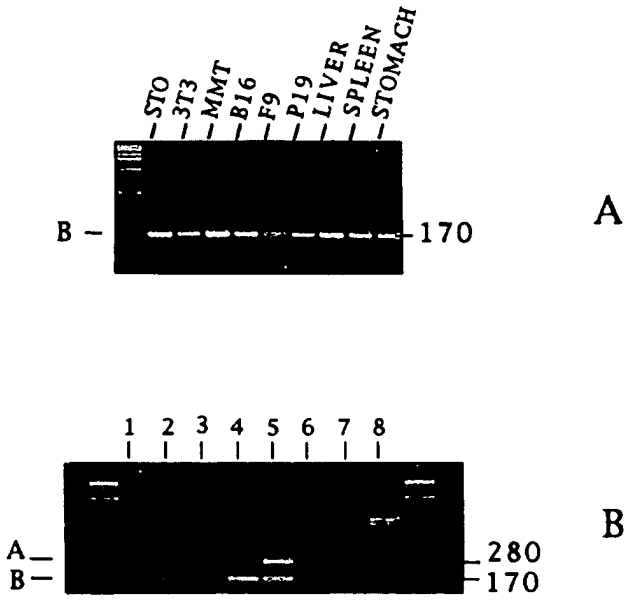


FIG. 5. *A*, RT-PCR amplification of mouse organ and cell line mRNAs. RT-PCR was performed on mRNA extracted from indicated mouse organs or cell lines, using mouse α7 primers 3154/3155. The 170-bp band corresponding to the α7B isoform is present in all samples. *B*, control RT-PCR. α7B plasmid amplified using 3154/3155 α7 primers (*lane 1*) or T3/T7 primers (*lane 2*); liver cDNA synthesized from 1 μg of mRNA (Clontech) and amplified with T3/T7 (*lane 3*) or 3154/3155 (*lane 4*), skeletal muscle cDNA from 1 μg of mRNA amplified with 3154/3155 (*lane 5*), H_20 (*lane 6*) and yeast mRNA (*lane 7*) amplified with 3154/3155 α7 primers, and yeast mRNA amplified with *Pyp3* yeast cDNA primers (*lane 8*, expected size 909 bp).

somehow yielded artifactual bands in the presence of reverse transcribed mRNA from any source, we amplified mRNA from the yeast *S. pombe*. No bands were produced with the α7 primers, whereas bands of the correct size were produced with primers for the yeast gene *Pyp3* (25).

Amplification of tissue cDNA with T3/T7 primers, which anneal to the plasmid vector pKs showed no product, indicating that there was no contamination of template with plasmid subclones (Fig. 5*B*).

Although we assumed a yield of 1–5 μg of poly(A)$^+$ mRNA/100 mg of tissue with the mRNA extraction kit (Invitrogen), in at least one experiment we started RT-PCR from formally identical amounts of mRNA. One μg each of mouse liver and skeletal muscle mRNA were reverse-transcribed and PCR-amplified with the α7 primers 3154/3155. Resulting bands were of approximately equal intensity to those observed in other experiments (Fig. 5*B*), making it unlikely that unusually high yields of mRNA in some tissue samples accounted for α7 detection.

Additionally, indirect evidence for the specificity of α7 detection was that although α7B PCR bands were found in all tissues and cell lines tested, bands corresponding to α7A remained detectable only in tissue or cells of skeletal muscle derivation (Figs. 4 and 5*B*).

Expression of α7 mRNA Isoforms during Muscle Differentiation—The above-mentioned results suggested ubiquitous expression of the α7B isoform mRNA (however, see "Discussion"). In contrast, the new α7A isoform mRNA appeared to be specific to skeletal muscle. Notably, however, the a7A isoform was absent in another type of muscle tissue, *i.e.* heart, which resulted α7A negative, α7B positive (Figs. 1 and 4). Because we could not be certain that the differential expression of α7 isoforms in heart *versus* skeletal muscle tissue homogenates reflected differences exclusively in the muscle cell components of these tissues, we tested myocardial cells in short term culture. These cultures were established from rat ventricular myocardium and contained less than 5% contaminating cell types. RT-PCR showed that cultured myocardial cells are α7B only (Fig. 4). Thus, α7 isoform differences between heart and skeletal muscle are likely due to the muscle cell component.

To determine whether the expression of α7A could be correlated with myoblast differentiation, fusion, and the formation of myotubes in skeletal muscle, we then tested the murine myoblast cell line C2C12, which has the ability to differentiate and form myotubes when cultured in appropriate medium (22). In the replicating (undifferentiated) state, these cells have the α7B isoform only (Fig. 4). After induction, the cells fuse to form multinucleated fibers. These differentiated myoblasts express both α7B and α7A isoforms (Fig. 4).

A time course experiment with C2C12 cells cultured under differentiation conditions is shown in Fig. 6*A*. In this experiment, the A/B heteroduplex band (see above) was faintly visible at 24 h, indicating the presence of a small amount of α7A. At 48 h, α7A was clearly visible and its quantity relative to α7B appeared steady thereafter (Fig. 6). In the same experiment, we also monitored the appearance of myogenin message (26), which became detectable by RT-PCR after 6 h of culture under differentiation conditions (Fig. 6*B*).

These results indicate that the new α7A isoform is expressed in skeletal muscle but not in cardiac muscle. Furthermore, α7A expression is concurrent with myoblast differentiation, and since it is detectable 18–36 h after myogenin, it is probably activated downstream of myogenin itself.

DISCUSSION

We have identified a structural variant of the α7 integrin subunit which differs from the previously published sequence in the cytoplasmic domain. Alignment of this new isoform with integrin α3A and α6A sequences shows 47.2 and 55.6% respective identity in the cytoplasmic region, indicating that it should be classified as α7A and the published sequence as α7B. Conserved motifs across the three A and B tails suggest a common evolutionary origin, perhaps by exon duplication in an ancestral gene. We also identified a highly conserved

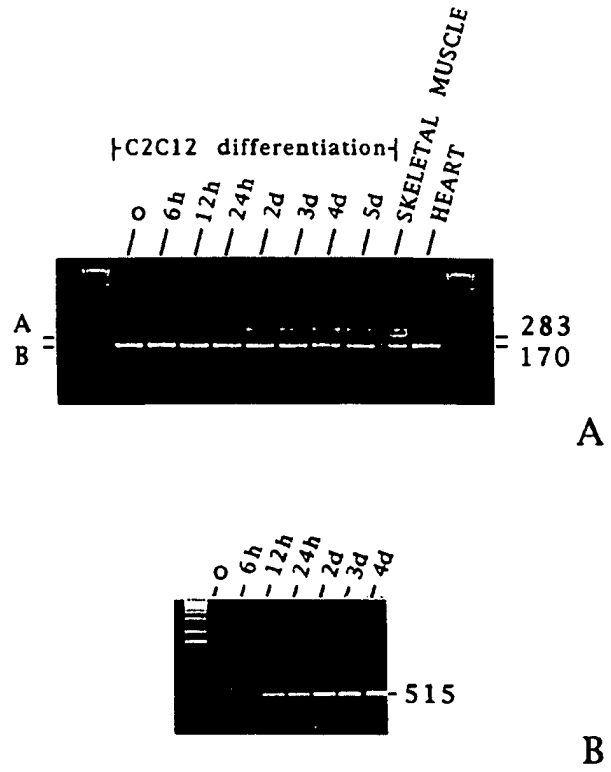


FIG. 6. *A*, α7A mRNA expression during C2C12 myoblast differentiation. RT-PCR was performed with 3154/3155 α7 primers on mRNA extracted from undifferentiated C2C12 myoblasts or after the indicated times of culture in differentiaton conditions. A band corresponding to the α7A message is visible at 48 h of culture and thereafter. Note that at 24 h a faint band corresponding to the position of the A/B heteroduplex (see text) is visible (*h* = hours; *d* = days). *B*, myogenin mRNA expression during C2C12 myoblast differentiation. Myogenin mRNA is detectable after 6 h, and its levels steadily increase with time. Note that the faint bands visible at time 0 represent background bands of unknown origin; none of these bands are of expected size (515 bp).

region with a distinct consensus within A and B tails, which we have termed nonamer box. This region may have functional value, perhaps interacting with distinct cytoplasmic components to mediate different cellular responses to common extracellular ligands.

The RT-PCR analysis we carried out on mouse organs and cell lines shows that the B isoform of α7 mRNA is present in all tissues and cells tested. Several controls substantiated this observation. For example, the possibility of plasmid contamination of mRNA templates or of primers were directly tested and eliminated (Fig. 5*B*). Furthermore, vast disproportion in the amount of starting mRNA obtained from tissues or cells could in principle give an equally disproportionate view of α7 expression. If, for example, mRNA yields from non-muscle tissues were consistently much higher than muscle tissues, the appearance of α7 bands in non-muscle tissues could be overestimated. When we started RT-PCR from equal amounts of liver and skeletal muscle mRNA, however, we obtained α7 bands of approximately equal intensity between the two samples, consistent with other experiments in which the quantity of starting tissue, rather than mRNA, was normalized. Thus, our data indeed suggest a widespread distribution for α7 mRNA (B isoform). This conclusion is surprising, in view of previous reports (14, 18, 21), indicating that α7β1 is expressed in few tissues or cell types, namely muscle cells or certain melanoma cell lines. However, in those instances α7 expression was detected by Northern blotting, by immunoprecipitation with monoclonal antibodies or by absorption/elution on laminin columns. All of these assays are inherently less sensitive than the RT-PCR analysis method. Furthermore, our RT-PCR assays were not quantitative, even though we estimated that the relative amount of α7 message amplification from various tissues was in the same range as skeletal muscle (Fig. 5*B*). Nonetheless, it is still possible that differences of an order of magnitude or more in mRNA abundance may be canceled out by the PCR amplification process, while producing negative *versus* positive results by Northern blotting. We also cannot be sure whether or not the mRNAs we detected are indeed translated into functional proteins or whether these proteins carry the epitopes recognized by current α7 monoclonal antibodies. More experiments are clearly required to clarify this issue.

Interestingly, we detected the α7A isoform mRNA only in skeletal muscle. RT-PCR may of course not be sensitive enough to determine with absolute certainty the complete absence of α7A expression in a particular sample. However, because the ratio of identically primed PCR products in a single reaction reflects the ratio of starting templates (27), it is possible in this instance to conclude that α7B mRNA is present in at least vast molar excess over α7A in all but skeletal muscle.

C2C12 replicating myoblasts contain only α7B as well, but 24–48 h after we induced differentiation, α7A became detectable. The appearance of the α7A mRNA splice variant also correlated with expression of myogenin in these cells. It has been suggested that α7 expression is differentially regulated at different stages of muscle development and that initial expression of α7 may precede and be independent from MyoD1 and myogenin expression (16). Our results confirm that C2C12 myoblasts express α7B at the undifferentiated stage. However, the α7A isoform becomes detectable 24 h after addition of differentiating medium or approximately 18 h after the appearance of myogenin. Thus, expression of the α7B integrin isoform seems to be independent of myogenin, whereas α7A expression seems to be located downstream of myogenin and may be a more accurate indication of differentiation of myogenic lines.

It has been shown that the overall level of α7 expression increases during myoblast differentiation and fusion (14) and that expression of α7 is regulated twice during skeletal muscle development at the formation of both primary and secondary fibers (16, 17). Appearance of an additional isoform of α7 during differentiation may contribute to these phenomena.

Laminin, but not fibronectin or collagen, is important to maintain myoblast proliferation and migration *in vitro* (19, 20). The binding of C2C12 replicating myoblasts to the E8 laminin fragment is mediated only by the α7β1 integrin (18). Because we identified α7A exclusively in skeletal muscle and in C2C12 during the process of differentiation but not during replication, it is possible that cellular responses to laminin may vary during differentiation according to which α7 isoform is being expressed, and two isoforms of the α7 laminin receptor in muscle cells may be necessary to regulate different events during muscle development. Expression of the α7A *versus* B tail may, in fact, influence the ability of cells to migrate on laminin. If this proves to be correct, it would be tempting to speculate that α3 and α6 integrins also perform similar functions in other cell types.

It has been suggested that α3, α6, and α7 subunits form a cluster with similar structures, functions, and modes of operation (6). The presence of alternative cytoplasmic domains in all of these subunits corroborates this view. α3, α6, and α7

are all subunits of laminin receptors which are often expressed in the same cell type. Such redundancy may have several explanations. It is possible that each binds to a distinct laminin isoform or that they have different affinities for the same binding sites on laminin. Our results strengthen the concept (6) that alternative cellular responses may be evoked through these receptors following cell attachment to laminin. For example, structurally diverse integrin cytoplasmic domains may allow such cell type-specific functions by interacting with cell type-specific effector molecules localized on the inner side of the plasma membrane. Splice variants are an ideal model to investigate this possibility in experimental terms.

Acknowledgments—We thank Dr. Silvia Evans for the gift of rat myocardium cells and for her helpful advice, Dr. Wanda Miller-Hance for the gift of myogenin primers, Dr. Charlie Glass for the gift of the C2C12 cell line, Dr. Clare McGowan for the gift of yeast RNA, and Dr. Jonathan B.A. Miller for the gift of *Pyp3* yeast cDNA primers. We acknowledge Barbra Blair for her expert assistance in the preparation of this manuscript.

REFERENCES

1. Ekblom, P., Vestweber, D., and Kemler, R. (1986) *Annu. Rev. Cell Biol.* **2**, 27–47
2. Buck, C. A., and Horwitz, A. F. (1987) *Annu. Rev. Cell Biol.* **3**, 179–205
3. Hynes, R. O., and Lander, A. D. (1992) *Cell* **68**, 303–322
4. Albelda, S. M., and Buck, C. A. (1990) *FASEB J.* **4**, 2868–2880
5. Ruoslahti, E. (1988) *Annu. Rev. Biochem.* **57**, 375–413
6. Hynes, R. O. (1992) *Cell* **69**, 11–25
7. Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G., and Silengo, L. (1990) *Gene (Amst.)* **95**, 261–266
8. Languino, L., and Ruoslahti, E. (1992) *J. Biol. Chem.* **267**, 7116–7120
9. van Kuppevelt, T. H. M. S. M., Languino, L. R., Gailit, J. O., Suzuki, S., and Ruoslahti, E. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5415–5418
10. Suzuki, S., and Naitoh, Y. (1990) *EMBO J.* **9**, 757–763
11. Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M., and Quaranta, V. (1990) *J. Cell Biol.* **111**, 1593–1604
12. Tamura, R. N., Cooper, H. M., Collo, G., and Quaranta, V. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10183–10187
13. Cooper, H. M., Tamura, R. N., and Quaranta, V. (1990) *J. Cell Biol.* **111**, 1593–1604
14. Song, W. K., Wang, W., Foster, R. F., Bielser, D. A., and Kaufman, S. J. (1992) *J. Cell Biol.* **115**, 843–850
15. Kaufman, S. J., Foster, R. F., Haye, K. R., and Faiman, L. E. (1985) *J. Cell Biol.* **100**, 1977–1987
16. Kaufman, S. J., George-Weinstein, M., and Foster, R. F. (1991) *Dev. Biol.* **146**, 228–238
17. Kaufman, S., J., and Foster, R. F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9606–9610
18. von der Mark, H., Durr, J., Sonnenberg, A., von der Mark, K., Deutzmann, R., and Goodman, S. (1991) *J. Biol. Chem.* **266**, 23593–23601
19. Foster, R. F., Thompson, J. M., and Kaufman, S. J. (1987) *Dev. Biol.* **122**, 11–20
20. Ocalan, M., Goodman, S. L., Kuhl, U., Hauschka, S. D., and von Der Mark, K. (1988) *Dev. Biol.* **125**, 158–167
21. Kramer, R. H., Vu, M. P., Cheng, Y. F., Ramos, D. M., Timpl, R., and Waleh, N. (1991) *Cell Regul.* **2**, 805–817
22. Yaffe, D., and Saxel, O. (1977) *Nature* **270**, 725–727
23. Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (1990) *PCR Protocol, A Guide to Methods and Application*, Academic Press, Inc., San Diego, CA
24. Starr, L., and Quaranta, V. (1992) *BioTechniques* **13**, 612–618
25. Miller, J. B. A., Lemaers, G., and Russell, P. (1992) *EMBO J.* **11**, 4933–4941
26. Wright, W. E., Sassoon, D. A., and Lin, V. K. (1989) *Cell* **56**, 607–617
27. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2725–2729

Journal of Cell Science 106, 1139-1152 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Expression of α_7 integrin cytoplasmic domains during skeletal muscle development: alternate forms, conformational change, and homologies with serine/threonine kinases and tyrosine phosphatases

Woo Keun Song, Weigwang Wang, Hiroyuki Sato, Deborah A. Bielser and Stephen J. Kaufman*

Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801, USA

*Author for correspondence

SUMMARY

We recently reported the cloning and sequencing of the α_7 integrin chain and its regulated expression during the development of skeletal muscle (Song et al. (1992) *J. Cell Biol.* 117, 643-657). The α_7 chain is expressed during the development of the myogenic lineage and on adult muscle fibers and this suggests that it participates in multiple and diverse functions at different times during muscle development. One interesting portion of this isoform is its cytoplasmic domain; comprised of 77 amino acids it is the largest in the alpha chains thus reported. In these experiments we begin to study the potential functions of the α_7 cytoplasmic domain by analyzing homologies between the rat and human sequences, by immunologic studies using an anti-cytoplasmic domain antiserum, and by identifying two alternate forms. In keeping with the nomenclature used to describe the α_3 and α_6 alternate cytoplasmic domains, we refer to the originally reported species as α_{7B} and the two additional forms as α_{7A} and α_{7C}. These three cytoplasmic domains likely arise as a consequence of alternate splicing. A splice site at the junctions of the transmembrane and cytoplasmic domains is used to generate the α_3, α_6 and α_7 A and B forms. The α_{7A} form RNA contains an additional 113 nucleotides compared to the B form, and a common coding region in the A and B form RNAs is used in alternate reading frames. Part of the coding region of α_{7B} appears to be used as the 3′-untranslated region of the α_{7A} form. The α_{7C} mRNA is 595 nucleotides smaller than the α_{7B} RNA and part of the 3′-untranslated region of the α_{7B} isoform is used as coding sequence in α_{7C}. There is developmental specificity in expression of these alternate mRNAs: α_{7A} and α_{7C} transcripts are found upon terminal myogenic differentiation whereas α_{7B} is present earlier in replicating cells and diminishes upon differentiation. We suggest this selective expression of the α_7 cytoplasmic domains underlies the diversity in function of the $\alpha_7\beta_1$ integrin at different stages of muscle development.

Immunochemical analyses indicate that the α_{7B} cytoplasmic domain undergoes a change in conformation in response to binding laminin or upon crosslinking initiated with antibody reactive with the integrin extracellular domain. Crosslinking also promotes association of the integrin with the cell cytoskeleton. Analysis of the amino acid sequence of the α_{7B} cytoplasmic domain reveals several motifs that may relate to the function of this protein. Two regions in the α_{7B} cytoplasmic domain have homology and similar apposition to those in the catalytic phosphotransfer domain and the ATP-binding site of serine/threonine protein kinases. There is also a sequence of 15 amino acids in the α_{7B} cytoplasmic domain that is homologous to that in many receptor-like protein tyrosine phosphatases. Although this cytoplasmic domain may be too small to have catalytic properties, it may effect the localization or regulation of these enzymes, or other proteins that interact with them. There is also a potential actin-binding sequence and a unique three-fold DXHPX repeat towards the carboxyl end of the α_{7B} cytoplasmic domain. Clearly, the α_{7B} cytoplasmic domain contains a rich potential for participating in the transduction of signals initiated outside the cell. This diversity in features, conformational changes, and forms of the α_7 cytoplasmic domains likely underlie its diverse functions on skeletal muscle.

Key words: integrin, muscle development, alternate splicing, cytoplasmic domains, protein kinase, phosphatase, laminin, cytoskeleton, myogenesis

INTRODUCTION

The integrins are a diverse family of heterodimeric cell surface integral membrane proteins that mediate the interactions of cells with each other, with extracellular matrix proteins, and either directly or indirectly, with additional molecules in their environment. The interactions of integrins with extracellular matrix proteins or with counter receptors

on other cells play significant roles in cell adhesion, migration and differentiation. The presence of integrins on most cells and their diversity of functions have made them a focus of study of many laboratories interested in cell, tissue and embryonic development, motility, cell structure and proliferation, clotting, and signal transduction, as well as specific pathologies associated with these processes, including metastasis and thrombosis (for reviews see, Albelda and Buck, 1990; Hemler, 1990; Springer, 1990; Hynes, 1992; Juliano and Haskill, 1993). The functional diversity exhibited by the integrins is largely due to the heterogeneity in integrin structure that is a consequence of the associations of alpha and beta chains that comprise the heterodimer. At least eight beta chains and fourteen alpha chains have been reported and the variety of heterodimers derived from these is believed to underlie the diversity in ligand binding and tissue specificity (Hynes, 1992). Preferred pairs of alpha and beta chains are expressed and associated in different cell types and more than one integrin is often expressed on individual cells.

Both alpha and beta integrin chains have a single long extracellular domain, a hydrophobic transmembrane domain, and (with exception of β4) a relatively short cytoplasmic domain. The capacity to bind ligand outside the cell is a function of both proteins. The sequences of the cytoplasmic domains of the different alpha and beta chain isoforms are quite divergent from one another: this is especially true among the alpha chains. Alternate forms of cytoplasmic domains that arise from alternate splicing have been found in the β_1, β_3, β4, α_3 and α_6 integrin cytoplasmic domains (van Kuppevelt et al., 1989; Altruda et al., 1990; Tamura et al., 1990, 1991; Cooper et al., 1991). Presumably, these diversities in the cytoplasmic domains also contribute to the varied capacities of the integrins to mediate the transduction of signals that initiate from extracellular interactions into the cell as well as signals that arise within the cell and are directed outward via activation of the integrins. The association of integrins with the cell cytoskeleton and the formation of adhesion plaques have been the most widely studied aspects of cytoplasmic domain functions and the beta chains seem to have a preeminent role. Some cytoplasmic proteins that mediate these interactions, for example vinculin and talin, have been identified (Springer and Paradiso, 1981; Horwitz et al., 1986; Otey et al., 1990) and phosphorylation of specific residues in the cytoplasmic domain appears to have a regulatory role (Hirst et al., 1986; Dahl and Grabel, 1989; Otey et al., 1990; Reska et al., 1992). Additional interactions of both alpha and beta chain cytoplasmic domains likely mediate integrin functions and these may be as diverse as the cytoplasmic domains themselves.

We recently reported the cloning and sequencing of the α_7 integrin chain and its regulated expression during the development of skeletal muscle (Song et al., 1992; George-Weinstein et al., 1993). The α_7 integrin also appears on some cells derived from the neural crest, including melanoma cells (Kramer et al., 1991), dorsal root ganglia and PC12 cells (S. J. Kaufman and M. George-Weinstein, unpublished data). The α_7 integrin is expressed on replicating secondary myoblasts (Kaufman and Foster, 1988; Kaufman et al., 1991; George-Weinstein et al., 1993). When these cells are grown on laminin, they undergo a change in shape, become more mobile and maintain their proliferation (Foster et al., 1987; Ocalan et al., 1988; Goodman et al., 1989). As the $\alpha_7\beta_1$ integrin is the sole functional laminin-binding integrin on these cells (von der Mark et al., 1991), we believe it underlies these behaviors and also functions to localize these cells at the laminin-rich sites of secondary fiber formation (George-Weinstein et al., 1993). Upon terminal differentiation there is an increase in the expression of α_7 on myotubes and this persists in adult fibers. α_7 localizes between adult fibers and the surrounding matrix (Song et al., 1992), and at myotendinous junctions where it likely serves to tether the fibers at its ends (M. George-Weinstein and S. J. Kaufman, unpublished results). The function of α_7 at these sites in adult muscle appears to be quite different from that on mobile, replicating myoblasts. These observations suggest that α_7 has multiple and diverse functions, and this raises the question, 'How are the requirements for these diverse functions resolved?'

One interesting characteristic of the α_{7B} isoform is its large cytoplasmic domain comprised of 77 amino acids, the largest of the alpha chains thus reported. In the experiments reported here we begin to study the potential functions of this cytoplasmic domain by analyzing homologies between the rat and human sequences, by immunologic studies using an anti-cytoplasmic domain antiserum, and by identifying two alternate forms of the α_7 cytoplasmic domain. One of these alternate forms, α_{7A}, is identical in amino acid sequence to that recently reported by Collo et al. (1993). The results of our experiments demonstrate multiple conserved and unique features of the α_7 cytoplasmic domain and conformational changes. This diversity in features, conformational states, and alternate forms, likely underlie the diversity in function of the α_7 integrin chain on skeletal muscle.

MATERIALS AND METHODS

Isolation of a human α_7 cDNA clone

A human fetal muscle λgt11cDNA library, kindly provided by Dr George Dickson, was screened by plaque filter hybridization using clone 05A rat α_7 cDNA (Song et al., 1992), labeled by the random priming method (Oligolabeling kit; Pharmacia) with [α-^{32}P]dCTP (Amersham; 3000 Ci/mmol). Hybridizations were performed at 65°C as described (Song et al., 1992), positive plaques were isolated, and recombinant phage DNA was purified from small scale plate lysates (Silhavy et al., 1984). cDNA fragments were subcloned in the *Eco*RI sites of pBluescript SK$^-$ and sequenced by the dideoxy chain termination method (Sanger et al., 1977).

Production of anti-α_7 cytoplasmic domain antiserum

A 27 amino acid peptide, NH_2-CEDRQQFKEEKTG-TIQRSNWGNSQWEG, was synthesized using t-BOC chemistry and an Applied Biosystems model 430A synthesizer, at the University of Illinois Biotechnology Center, and purified using a Vydak C-18 reverse phase chromatography column, eluted with a 0-60% gradient of 0.1% trifluoroacetic acid (TFA) and 0.1% TFA/70% acetonitrile. This peptide (excluding the N-terminal cys-

teine) is within the α_{7B} integrin cytoplasmic domain. The peptide was further purified using a Bio-Rad 10 DG exclusion column. 6.7 mg of peptide in 300 μl phosphate buffered saline (PBS), was added to 10 mg of maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce) in 10 ml PBS and incubated at room temperature for 2 hours. Then 100 μl of 100 mM cysteine was added for 15 minutes and the protein was dialyzed against 500 ml PBS overnight at 4°C. New Zealand White rabbits were immunized at two intramuscular and subcutaneous sites with approximately 1 mg of conjugated protein emulsified in an equal volume of Ribi adjuvant (Ribi Immunochemical). Each rabbit was immunized 7 times over five months. The serum was delipified with 0.25% sodium dextran sulfate and 1% $CaCl_2$, centrifuged at 10,000 *g* for 10 minutes, and the antibody was precipitated with 50% ammonium sulfate and dialyzed extensively against PBS. Immunoblot analyses using the peptide coupled to bovine serum albumin (BSA) demonstrated the specificity of the respective sera for the immunizing peptide.

Immunofluorescence

L8E63 myogenic cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% horse serum (Gibco), on 12 mm glass coverslips coated with 0.1% gelatin as described (Kaufman and Parks, 1977). Cultures of cells from the thighs of newborn rat hindlimbs were prepared and grown as indicated (Foster et al., 1987). Cells crosslinked prior to staining with anti-cytoplasmic domain antibody (anti-CD) were first reacted either with anti-α_7 integrin or anti-laminin 2E8 (Engvall et al., 1986; Developmental Hybridoma Bank) monoclonal antibodies followed by fluorescein-conjugated donkey anti-mouse immunoglobulin (Jackson Immunoresearch). These cells were then fixed with 95% ethanol or treated with 0.25% Triton X-100 for 10 minutes. Staining was then done with anti-CD antibody (a 1:200 dilution of a 50% ammonium sulfate cut), followed by rhodamine-conjugated donkey anti-rabbit immunoglobulin (Jackson Immunoresearch). After staining, the cells previously extracted with Triton X-100 were fixed with 95% ethanol. Live cells were reacted for 5 minutes with laminin diluted to 1, 10 or 100 μg/ml in Dulbecco's PBS (DPBS) containing 0.1% gelatin. These cells were immediately fixed with 95% ethanol and stained with anti-CD antibody. To demonstrate specificity, the anti-CD antibody was blocked with α_7 cytoplasmic domain peptide by incubating equal volumes of antibody and 200 μg/ml peptide for 30 minutes. The antibody was then diluted to the appropriate concentration for staining. To determine the effect of temperature on promoting accessibility of the cytoplasmic domain, individual coverslips in dishes were maintained at the specified temperatures for 5 minutes. The cells were immediately fixed with 95% ethanol at room temperature. The coverslips were mounted in glycerol/PBS (9/1, v/v), pH 8.5, containing 10 mM *p*-phenylenediamine (Eastman), sealed with Flo-texx (Fisher), and examined with a Zeiss photomicroscope III equipped with epi-illumination optics and an HBO 100 W mercury lamp.

Immunoblotting

Cell lysates were made from cells rinsed three times with DPBS, then with DPBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then scraped with a rubber policeman and pelleted by centrifugation. The pellets were resuspended in PBS containing 1 mM PMSF and sonicated three times, for 5 seconds using a Branson 200 Sonifier, set at output 5 and 50% duty cycle. The lysates were then electrophoresed in 8% SDS-polyacrylamide gels at 40 mA. The gels were equilibrated in 25 mM Tris, 200 mM glycine, pH 8.8, and 20% methanol and transferred onto nitrocellulose at 100 V, for 60 minutes. The blots were rinsed at room temperature for 60 minutes in TSTB (5 mM Tris, 75 mM NaCl, and 0.5% Tween-20, pH 7.5) containing 2% gelatin. The nitrocellulose was then rinsed 3 times, 10 minutes each, in TSTB containing 0.5% gelatin and then reacted with the appropriate antibodies diluted in this same buffer. After rinsing, the blots were reacted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Jackson Immunoresearch) for 60 minutes, washed, and developed with nitro blue tetrazolium (NBT, 55 mg/ml in 70% *N,N*-dimethylformamide) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 mg/ml in 100% *N,N*-dimethylformamide) in 10 ml 150 mM NaCl, 5 mM EDTA, and 100 mM Tris-HCl, pH 9.5. The blots were then rinsed with TSTB, then with 20 mM Tris, pH 2.9, 1 mM EDTA, and allowed to air dry. Prestained molecular mass markers were included in each gel.

Deglycosylation of α_7 integrin

L8E63 myotubes, on three 100 mm dishes, were collected using a rubber policeman, pelleted and extracted twice, for 30 minutes, at 4°C, with 100 μl of extraction buffer (200 mM octyl-β-*D*-glucopyranoside, 1 mM PMSF and 100 mM Tris-HCl, pH 7.4). For deglycosylation, SDS was added to a final concentration of 1%, the extract was then boiled for 2 minutes, then adjusted to 20 mM sodium phosphate buffer, pH 7.2, 10 mM sodium azide, 50 mM EDTA and 0.5% NP-40, and boiled for 2 minutes. The denatured cell extract was then incubated with peptide-*N*-glycosidase F (PNGase, 20 mU/50 μl cell lysate, Boehringer Mannheim) at 37°C for 18 hours and analyzed in immunoblots.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed as described (Song et al., 1992). Single-stranded cDNA was synthesized using 100 ng poly(A)$^+$ RNA purified from L8E63 myoblasts or myotubes, or 1 μg total RNA extracted from L8E63 cells, fu-1 cells or newborn myoblasts grown as indicated (Foster et al., 1987), 15 units of AMV reverse transcriptase (Promega) and 0.2 μg of oligo (dT) primer. The single-stranded cDNAs were amplified in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.4) containing 1.0 μM of 17mer sense primer (5′-AGCCGTGCTTCATGTCT-3′) and 1.0 μM 17mer antisense primer (3′-GGCTGGTACAGCACACT-5′) for amplification of the mRNAs encoding α_{7A} and α_{7B} cytoplasmic domains, or, 1.0 μM of 20mer antisense primer (3′-TGGTACAGCACACTTGAAGA-5′) for amplification of the α_{7C} mRNA, 250 μM each of dATP, dCTP, dTTP and dGTP, and 1.25 units/50 μl reaction mixture of *Taq* polymerase (Promega). The samples were cycled 30 times at 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 1.5 minutes. The PCR products were separated on 0.8% agarose gels, the fragments were cut out, purified using GeneClean II (Bio101) and cloned using the ddT-tailed vector system described by Holton and Graham (1990). Then 5 μg of pBluescript SK$^-$ was digested with *Eco*RV and was tailed in 25 mM Tris-HCl, pH 6.6, 200 mM potassium cacodylate, 250 μg/ml BSA, 1.5 mM $CoCl_2$ and 10 μM ddTTP (Pharmacia) using 50 units of terminal transferase (Promega), for 1 hour at 37°C. ddT-tailed plasmid was purified with phenol/chloroform, followed by ethanol precipitation, and ligated with the purified PCR fragment using T4 DNA ligase (BRL). XL-1 cells were transformed with the ligation product, plated on X-gal and white colonies were selected.

Computer analyses of protein sequences

Protein sequence homology searches were carried out using the BLAST programs developed by National Center for Biotechnology Information at the National Library of Medicine (Altschul et al., 1990). Protein secondary structure analyses were performed with the algorithms of Chou and Fasman (1974), Garnier et al.

(1978), and Hopp and Woods (1981), using MacVector 3.0 (IBI, New Haven, CT).

RESULTS

Alternate forms of the α_7 cytoplasmic domain

Reverse-transcriptase PCR analysis, using paired primers specific for the cytoplasmic domain of the α_7 integrin, revealed two products. This suggested that two α_7 isoforms were expressed during the differentiation of the L8E63 myogenic cell line (Fig. 1). In contrast with the 899 bp product detected throughout the course of myogenesis in vitro, a 113 nucleotide larger fragment was generated from poly(A)$^+$ RNA prepared from cells in cultures that had undergone differentiation. The 1012 bp fragment was cloned into pBluescript KS$^-$ and its nucleotide and inferred amino acid sequences were determined. The larger PCR fragment represents an mRNA that encodes an alternate 58 amino acid cytoplasmic domain. In keeping with the nomenclature previously used for isoforms of the α_3 and α_6 cytoplasmic domains, and the homologies of the respective A and B isoforms, this species has been referred to as α_{7A} and the original form as α_{7B}. An alternate isoform of the mouse α_7 cytoplasmic domain was recently cloned and sequenced by Collo et al. (1993). The sequence of the rat α_{7A} cytoplasmic domain (Fig. 2A) differs at five nucleotides from that of the mouse, and there is a single residue difference in the 58 amino acid sequence. A common coding region in the A and B form RNAs is used in alternate reading frames, and part of the coding region of α_{7B} is in the 3′-untranslated region of the α_{7A} RNA. Two splice sites appear to be located 5′ to the GFFKR coding regions of the A and B form RNAs.

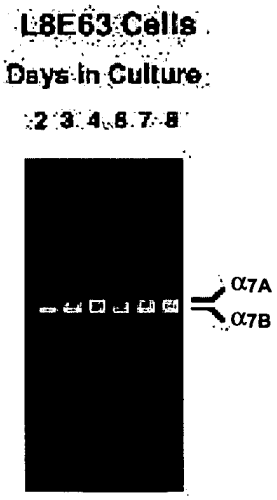


Fig. 1. Alternate forms of the α_7 cytoplasmic domain. Cells from the L8E63 myogenic cell line were cultured in vitro for 2 to 8 days. Poly(A)$^+$ RNA was isolated and subjected to RT-PCR. The amplified products were analyzed by gel electrophoresis and ethidium bromide staining. Two species of amplified product were detected: α_{7A} mRNA produces a 1012 bp band, while α_{7B} mRNA produces a 899 bp fragment.

An additional alternate isoform of the α_7 cytoplasmic domain was detected in a lambda UniZap rat muscle cDNA library screened with a 293 nucleotide *Pst*I fragment produced from the 5′-end of the 05B α_7 cDNA clone. This fragment encodes a 97 amino acid region in the extracellular domain that is unique to the α_7 integrin. Forty-five clones were identified and inserted into the pBluescript SK$^-$ plasmid by in vivo excision and transformation into XL-1 blue cells. Plasmid from these cells was pooled into nine groups, each representing five positive clones. The DNA sequences bounded by the 17mer sense and antisense primers (Fig. 2B) were amplified using *Taq* polymerase. Two fragments were generated, one 899 bp, representing the amplified fragment of the α_7 cDNA previously reported (Song et al., 1992), and a second, 304 bp fragment. Individual clones in the positive groups were analyzed by PCR amplification and two clones containing the shorter fragment were identified (Fig. 3A) and sequenced from their 3′-ends. The 5′-ends of these sequences, which encode the membrane spanning region and extend into the extracellular domain, were identical with the sequence of α_{7B} (Fig. 2B). A deletion of 595 nucleotides in α_{7B} accounts for the shorter sequence, which we refer to as α_{7C}. A new open reading frame is generated as a consequence of this deletion and results in the alternate protein sequence in the cytoplasmic domain that begins with a switch from the GFFKR sequence in α_{7B} to GFFKC in α_{7C}. Part of the 3′-untranslated region present in the α_{7B} RNA is used to encode the α_{7C} form and a new termination codon, TAA (Fig. 2).

In contrast with the 58 amino acids in the α_{7A} form and 77 amino acids in the α_{7B} cytoplasmic domain, the α_{7C} cytoplasmic domain contains 18 amino acids. Potential sites of phosphorylation in the three α_7 cytoplasmic domains were determined using reported consensus sequences (Aitken, 1990). As indicated in Fig. 2C, each cytoplasmic domain has a single site of potential tyrosine phosphorylation and this appears to be the sole potential phosphorylation site in the α_{7B} cytoplasmic domain. The α_{7A} and α_{7C} domains each have a serine residue that may be phospho-

Fig. 2. Alternate splicing produces three transcripts encoding three forms of the α_7 cytoplasmic domain with different potential phosphorylation sites. The deduced α_{7A}, α_{7B} and α_{7C} cytoplasmic domains are 55, 77 and 18 amino acids, respectively. (A) The α_{7A} transcript is 113 nucleotides longer than the α_{7B} and a common coding region in α_{7A} and α_{7B} is used in alternate reading frames. Part of the coding region of α_{7B} appears to be used as the 3′-untranslated region of the α_{7A} form. The amino acid sequence of the α_{7A} cytoplasmic domain is shaded. (B) The α_{7C} transcript is 595 nucleotides shorter than the α_{7B} form. The 3′-untranslated region of the α_{7B} form is used as coding sequence for the α_{7C} cytoplasmic domain. Filled boxes indicate the transmembrane coding regions (underlined), open boxes indicate the coding regions for cytoplasmic domains, and boxes with diagonals refer to the 3′-untranslated regions. The translation stop codons (TLS) are indicated by arrows. The PCR primers used to detect the alternate RNAs are indicated by the bold arrows. (C) The potential sites of phosphorylation of the α_7 cytoplasmic domains are indicated: ▼, tyrosine protein kinase site; •, calcium, calmodulin dependent protein kinase II site; ◆, cyclic GMP dependent protein kinase site; ●, protein kinase C site.

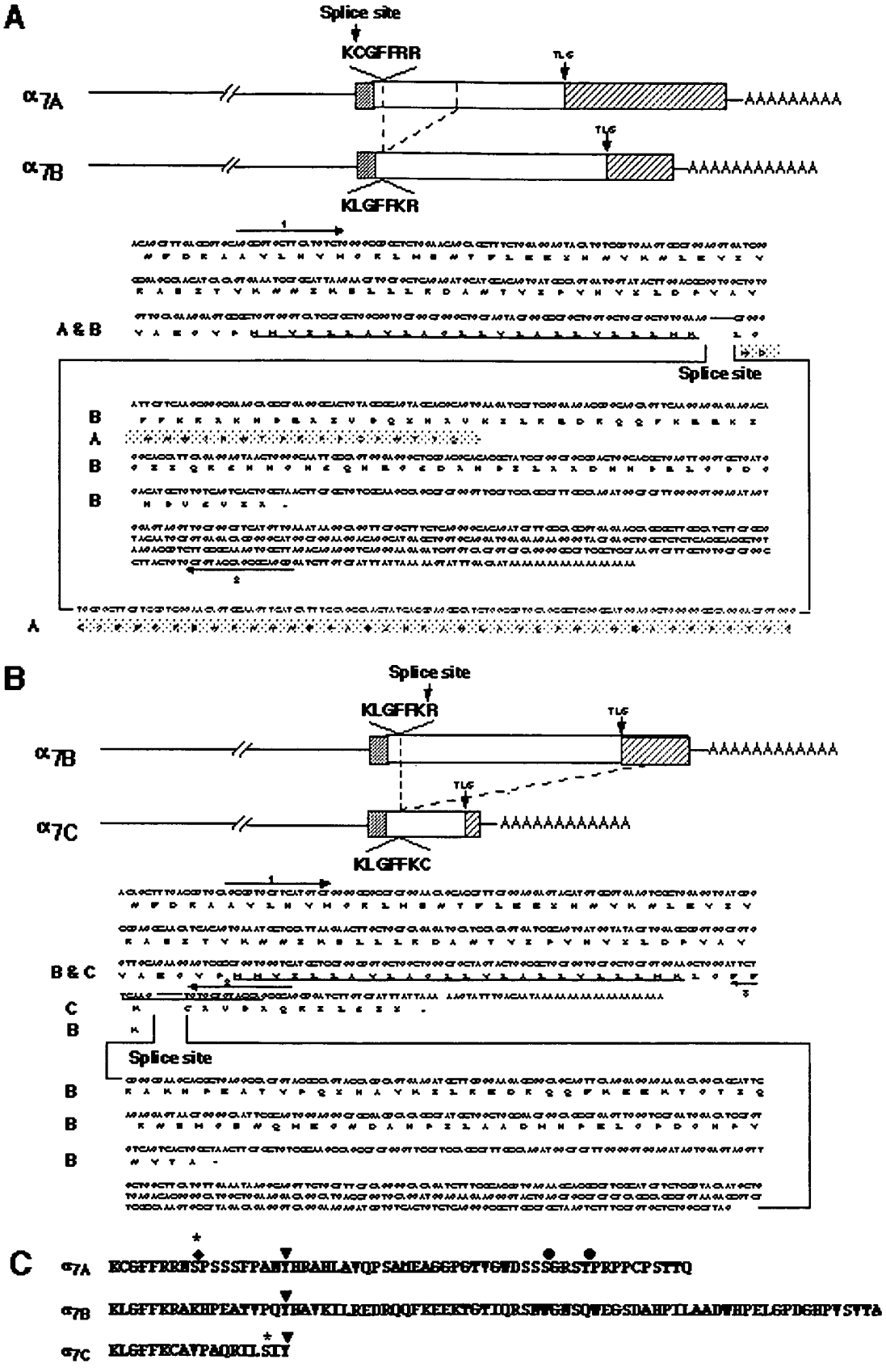

A
Splice site
KCGFFRR
TLG
α7A
AAAAAAAAAA
α7B
TLG
AAAAAAAAAAAA
KLGFFKR
A & B
Splice site
B
A
B
B
A
B
Splice site
KLGFFKR
TLG
α7B
AAAAAAAAAAAA
TLG
α7C
AAAAAAAAAAAA
KLGFFKC
B & C
C
B
Splice site
B
B
B
C
α7A
α7B
α7C

Fig. 2

rylated by calcium-, calmodulin-dependent protein kinase II: this same serine in α_{7A} may be phosphorylated by cGMP-dependent protein kinase. An additional serine and threonine residue in the α_{7A} cytoplasmic domain may be a substrate for phosphorylation by protein kinase C.

Expression of the α_7 cytoplasmic domains is developmentally regulated

The differentiation of primary cultures of myogenic cells in vitro more closely resembles in vivo development and these cells were used to confirm that the expression of the α_7 cytoplasmic domain isoforms was developmentally regulated. RNA was isolated from cultures of differentiating newborn skeletal myoblasts at various times after these cells were placed in culture. The medium in the cultures was switched on day 2 to one that promotes differentiation. As seen in the results of RT-PCR analysis, expression of α_{7A} accompanies myogenic differentiation whereas α_{7B} is present earlier, in replicating myoblasts, and diminishes upon differentiation (Fig. 4). fu-1 cells, a developmentally defective line derived from L8E63 myoblasts, which have lost their normal control of proliferation, do not differentiate and are transformed and tumorigenic (Kaufman and Parks, 1977). Immunofluorescence (Kaufman et al., 1985) and northern analyses demonstrate that these cells express reduced α_7 integrin (Song et al., 1992) and as shown here using RT-PCR, fu-1 cells express the α_{7B} but not the α_{7A} integrin chain (Fig. 4). This supports the conclusion that the shift in expression of α_{7B} to α_{7A} is part of the developmental process that accompanies the differentiation of skeletal muscle.

To detect the mRNA that encodes the α_{7C} cytoplasmic domain RT-PCR was performed using an antisense primer that is specific to this form (Fig. 2A) and poly(A)$^+$ RNA purified from L8E63 cells. The fragment produced by PCR was ddT-tailed, cloned into the pBluescript SK$^-$ plasmid and sequenced. This nucleotide sequence was identical to that obtained from the cDNA library, confirming the expression of this alternate form of the α_7 protein. RT-PCR detected the α_{7C} mRNA in poly(A)$^+$ RNA prepared from myotubes but not myoblasts (Fig. 3B). This isoform of α_7 mRNA appears to be present in relatively low amounts as it was detected only after two rounds of PCR.

These three forms of the α_7 chain likely originate by alternate RNA splicing. The structures of the mRNAs that are consistent with the procedure used to generate these cDNAs are indicated in Fig. 2. We suggest that part of the diversity in function of the α_7 integrin at different stages of muscle development is due to the developmentally regulated expression of the alternate cytoplasmic domains. Additional studies on the α_{7B} cytoplasmic domain were undertaken to further define its function in replicating myoblasts.

Comparison of the human and rat α_{7B} cytoplasmic domains

A 1.9 kb clone of the 3′-end of α_7 cDNA was identified in a cDNA library prepared from human fetal muscle, isolated, and sequenced. The nucleotide coding regions and inferred amino acid sequences of the rat and human α_{7B} cytoplasmic domains were determined to be 84% identical (Fig. 5). Two regions in the α_{7B} integrin cytoplasmic domain are

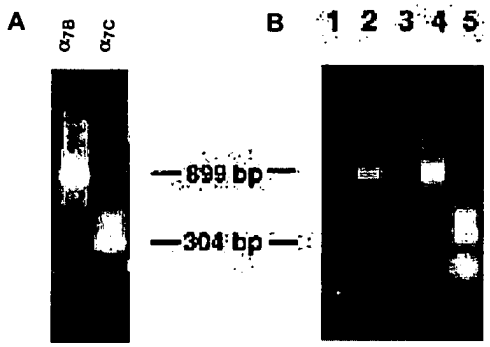


Fig. 3. Identification of α_{7B} and α_{7C} cDNAs and mRNAs by PCR. (A) PCR was performed on forty-five α_7 cDNA clones using the sense (1) and antisense (2) primers (indicated by arrows in Fig. 2). Two clones produced a 304 nucleotide bp fragment and were sequenced and identified as α_{7C}. The PCR fragment produced from the α_{7B} form was 899 bps: the C form represents a 595 bp deletion of the B form. (B) α_{7B} and α_{7C} mRNAs were detected in poly(A)$^+$ RNA isolated from L8E63 myoblasts (lanes 2 and 3) and myotubes (lanes 4 and 5) by RT-PCR with primers 1, 2 and 3 (Fig. 2). The α_{7B} 899 bp band (lanes 2 and 4) was produced after one round of PCR using primer set 1 and 2. The α_{7C} 304 bp fragment was evident after a second round of PCR using myotube (lane 5) but not myoblast RNA (lane 3) using primer set 1 and 3. Lane 1, control: no RNA; primer set 1 and 3.

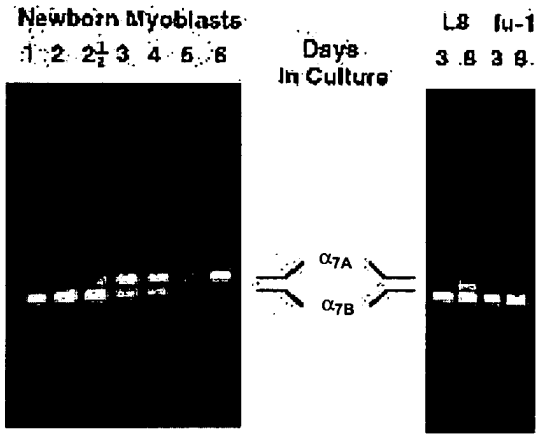


Fig. 4. Expression of α_{7A} and α_{7B} integrin is developmentally regulated. Cells from the newborn rat hindlimb were initially cultured in growth medium (10% fetal bovine serum plus 10% horse serum) and on day 2 the medium was changed to 10% horse serum to facilitate differentiation. L8E63 cells and developmentally defective mutant fu-1 cells were grown for 3 or 8 days. Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction at the times indicated and subjected to RT-PCR. The amplified products were analyzed by gel electrophoresis and ethidium bromide staining. A switch from expression of α_{7B} to α_{7A} accompanies myogenic development in vitro. fu-1 cells do not differentiated and do not express α_{7A}.

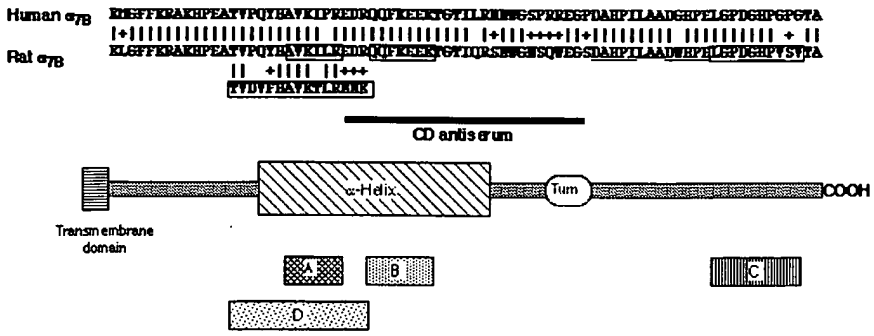


Fig. 5. Structural analysis of the α_{7B} integrin cytoplasmic domain. The amino acid sequence of the human α_{7B} integrin cytoplasmic domain was determined and aligned with the rat α_{7B} sequence. Identical residues (|) and conserved changes (+) are indicated. α-Helix and turn regions are predicted by MacVector analysis. BLAST analysis of the sequences with existing data banks in the National Center for Biotechnology Information at the National Library of Medicine indicates several potential motifs in the rat α_{7B} cytoplasmic domain. A, a phosphotransfer motif and C, an ATP-binding motif common to serine/threonine kinases. B, a potential actin-binding region similar to that in villin. D, a hydrophobic sequence common to receptor-like protein tyrosine phosphatases. A threefold DXHP repeat, unique to this protein, is underlined in the rat α_{7B} sequence. The bold line indicates the sequence of 26 amino acids in α_{7B} used to prepared an anti-cytoplasmic domain (CD) antiserum.

highly conserved between the human and rat sequences: these are the 32 amino acids directly on the carboxyl side of the GFFKR sequence (97% indentity between species) and the sequence of 21 amino acids that begins 4 amino acids from the carboxyl terminus and contains a DXHPX repeat (discussed below). All four threonine residues are maintained at the identical positions in the cytoplasmic domains. These identities in the human and rat α_{7B} cytoplasmic domains suggest that this integrin alpha chain has the same function in different species, and that the highly conserved portions likely play a significant role in mediating this function.

Structural motifs in the α_{7B} cytoplasmic domain

α-Helix

Computer analysis (Chou and Fasman, 1974; Garnier et al., 1978) of the α_{7B} cytoplasmic domain sequence suggests an α-helical conformation comprised of approximately 25 amino acids (Fig. 5).

Serine/threonine protein kinase homology regions

The sequence, AVKIL(P)R, is present in the α-helical region of the α_{7B} cytoplasmic domain. This same sequence is conserved in subdomain II, the catalytic site of serine/threonine protein kinases. Site-directed mutagenesis of the lysine residue (K) within this sequence results in the loss of protein kinase enzymatic activity (Hanks et al., 1986). The ATP-binding domain of these protein kinases have the consensus sequence LGXGXXGXV. The rat α_{7B} sequence, LGXXGXXVXV, located towards the carboxyl end of the cytoplasmic domain, shares this homology. The human sequence, LGXXGXXGXG, also shares this homology.

DXHPX repeats in the α_7 cytoplasmic domain

The sequence Asp/X/His/Pro/X, where X is a hydrophilic residue, is repeated three times in the α_{7B} cytoplasmic domain. The most carboxyl repeat is within the potential ATP-binding site cited above. These repeats, and the three amino acids that separate them, are conserved 95% between the human and rat α_{7B} integrins. The DXHPX repeat in the α_{7B} cytoplasmic domain is unique amongst integrin alpha chains, moreover, it was not found in any sequence in current protein or nucleic acid data banks.

Receptor-like protein tyrosine phosphatase homology region

The α–helical region in the cytoplasmic domain also contains a sequence of 15 amino acids, TVPQYHAVK-ILREDR, that is 80% homologous with a region in the family of receptor-like protein tyrosine phosphatases (Gebbink et al., 1991). As noted above, the AVKIL motif within this sequence is also common and essential to the enzymatic activity of protein kinases. A sequence with 92% homology to PQYHAVKILREDR is also present in the cytoplasmic domains of the human α_{3B} and α_{6B} integrins, however, the lysine residue essential to protein kinase activity has been replaced by arginine in α_{3B} and α_{6B} whereas it is maintained in the human and rat α_{7B} (Fig. 6).

Putative actin-binding site

A potential actin-binding site, QQFKEEK, that closely resembles the actin-binding site of villin (QQNLKKEK), is also found within the α-helix region. This sequence in villin is also within an α-helical domain, and mutation to QQN-LKEEK, which resembles α_7 integrin even more closely, still has 80% actin-binding capacity (Friederich et al., 1992). The other two lysine residues are essential for actin-binding.

Structural changes in the α_{7B} integrin cytoplasmic domain

A 26 amino acid segment of the cytoplasmic domain of the rat α_{7B} chain (Fig. 5) was synthesized with an additional cysteine residue at the amino terminus. This portion of the cytoplasmic domain was chosen because its sequence is unique from that of other reported alpha chains and its potential high antigenicity (Hopp and Woods, 1981). The antiserum was evaluated for reactivity with α_7 integrin by immunofluorescence and immunoblot analyses.

The major polypeptides in unreduced extracts of L8E63 myoblasts and myotubes that were reactive after electrophoresis in 8% polyacrylamide gels had mobilities cor-

Homology of α_7 Cytoplasmic Domain

KCDFFKPTRTYYRPIHPKYHAVRIREEER α_{3B} (mouse)
KCGFFKRSRY-DDSVPRYHAVRIRKEER α_{6B} (human)
KLGFFKRAKHPEATVPQYHAVKILREDRQQFKEEKTGTIQRSNWGNSQWEGSDAHPILAADWHPELGPDGHPVSVTA α_{7B} (rat)
TV +HAVK LR+++
TVDVFHAVKTLRNNK Receptor-like Protein Tyrosine Phosphatase (rat RPTP)

Comparison of α_7 Integrin with Protein Tyrosine Phosphatase Type Proteins

VPQYHAVKILR	α_7 Integrin (rat)
VDVFHAVKTLR	RPTP (rat)
VDVFQVVKSLR	Leukocyte common antigen (rat)
VDMFQTVKTLR	Leukocyte common antigen related (human)
VDIFQVVKALR	CD45 (rat)
VDIFQTVKMLR	PTP-δ (human)
LDVFQAVKSLR	PTP-ε (human)
LDVFQTVKSLR	RPTP (mouse)
LDVFQTVRILR	PTP (*Drosophila*)

Fig. 6. Comparison of the receptor-like protein tyrosine phosphatase homology regions in the α_{3B}, α_{6B}, and α_{7B} cytoplasmic domains. A region in the α_{7B}, α_{3B}, and α_{6B} integrin cytoplasmic domains has homology to a sequence in receptor-like protein tyrosine phosphatases (RPTP). Identical amino acids in the conserved region of these three integrin alpha chains are indicated in bold. Identical amino acids are printed between the rat α_7 integrin and the rat RPTP sequences. The plus (+) signs show conserved changes of amino acids. Homology with other RPTP proteins is also indicated.

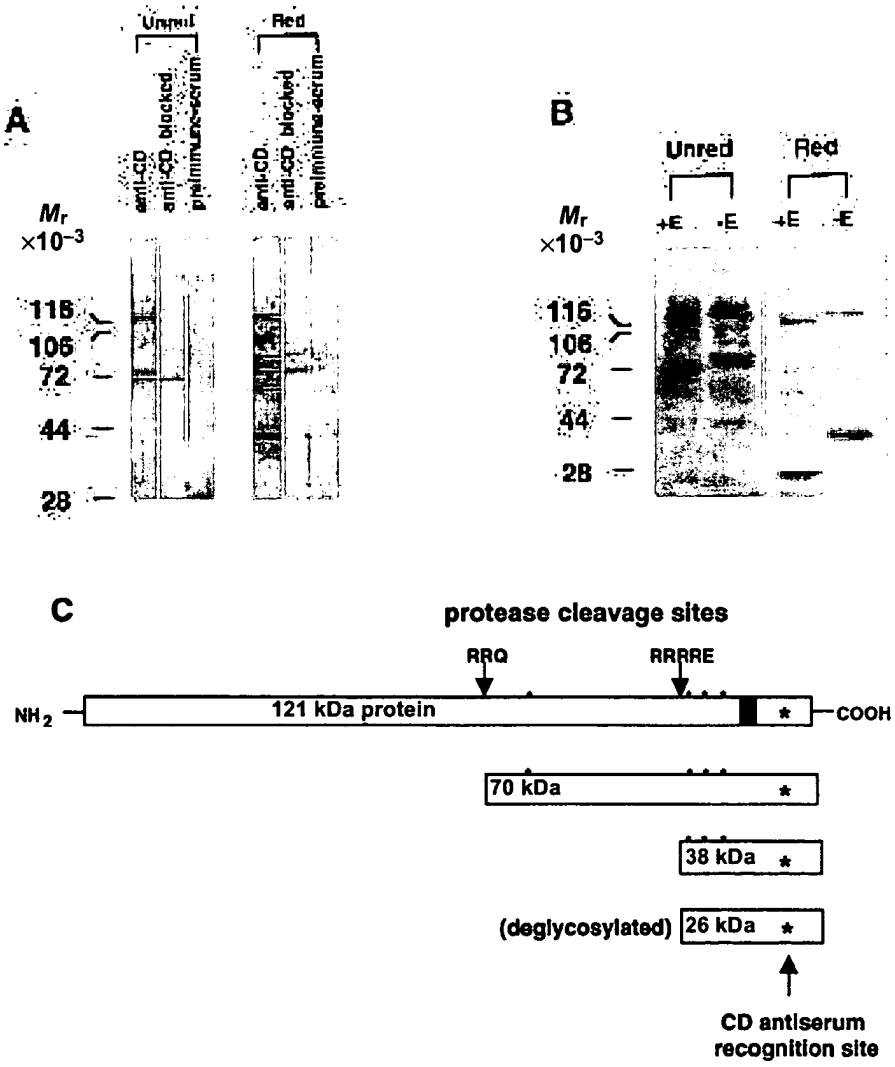


Fig. 7. Anti-α_{7B} cytoplasmic domain antibody detection of intact α_7 chain and its proteolytic cleavage products is specific and demonstrates glycosylation of the alpha chain. (A) Immunoblots of unreduced and reduced L8E63 cell lysates. Specific binding was blocked by preincubation of the anti-CD antibody with the immunizing peptide. (B) L8E63 cell lysates were treated with endoglycosidase F to deglycosylate the protein and electrophoresed under reduced or nonreduced conditions. The migration of the deglycosylated form (26 kDa) corresponds to the molecular mass determined from the amino acid composition. (C) The protease cleavage sites in the α_{7B} chain and the corresponding peptides detected in the immunoblots are indicated.

responding to approximately 121,000 and 70,000 Da. These two bands represent the intact α_7 chain and a product of one of two cleavage sites in the molecule. Upon reduction, the predominant band reactive with this antiserum migrated at approximately 38,000 Da (Fig. 7A). This peptide represents the carboxyl terminal peptide that can originate either from a single proteolytic cleavage of the intact α_{7B} chain at the RRRRE site or from the 70,000 Da peptide (Fig. 7C). A small amount of the 121,000 Da protein persisted, indicating that all the α_{7B} in the extract had not undergone proteolytic cleavage. The molecular mass of the 38,000 Da peptide deduced from its amino acid sequence is 26,000 Da

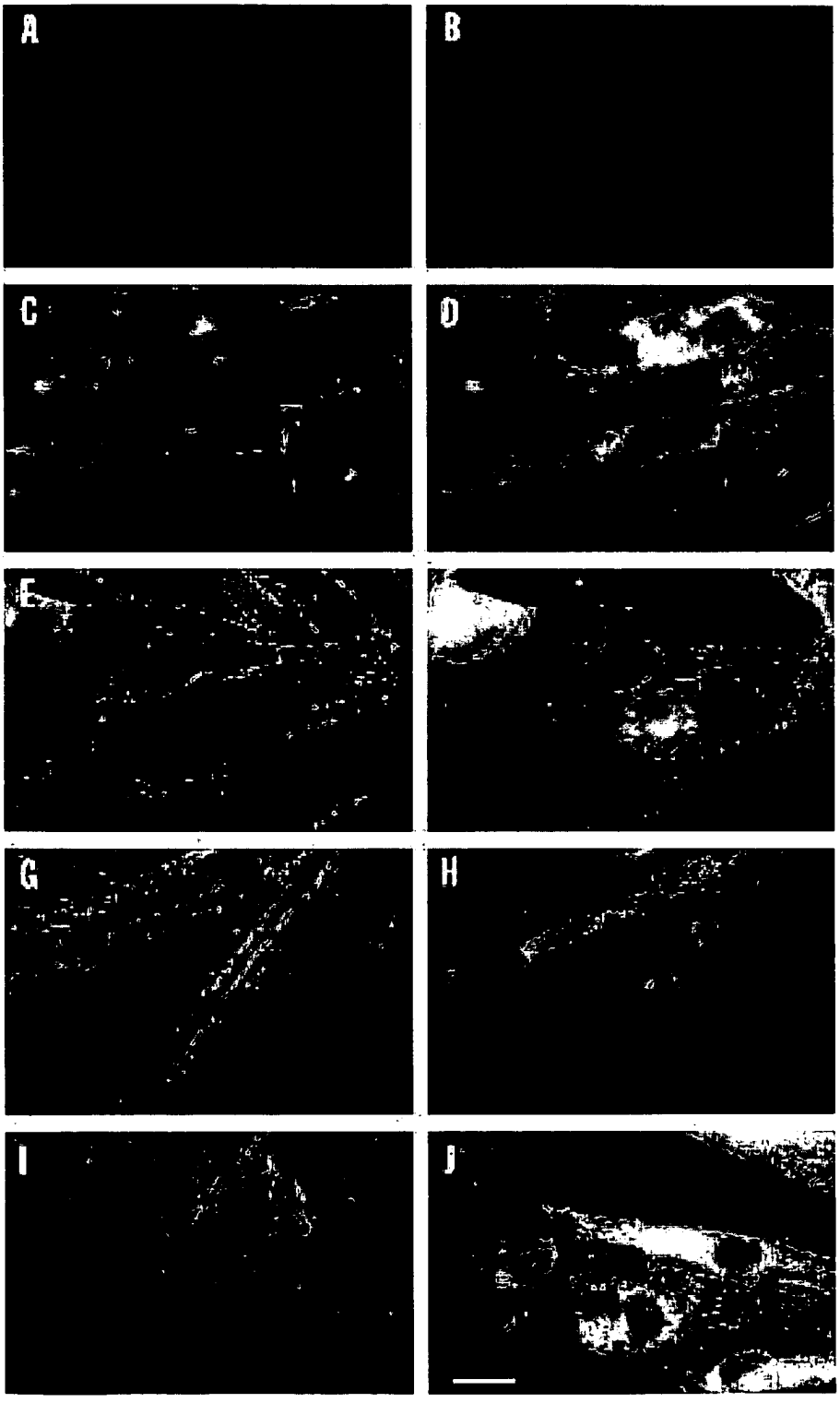


Fig. 8. Crosslinking the extracellular domain of the α_{7B} integrin with antibody or binding ligand promotes a conformational change in the cytoplasmic domain and association with the cell cytoskeleton. When L8E63 cells were fixed with ethanol (A), or extracted with Triton prior to addition of antibody (B), little immunofluorescence was detected by anti-CD antiserum. Upon crosslinking the extracellular domain with primary and secondary antibodies, or after addition of laminin, the cytoplasmic domain becomes accessible to anti-CD antiserum and the complex becomes associated with the Triton X-100-insoluble cell cytoskeleton. Cells were reacted with H36-α_7 monoclonal antibody (C), followed by fluorescein-conjugated donkey antimouse IgG, fixed with ethanol and stained with anti-CD antibody and rhodamine-conjugated goat antirabbit IgG (D). Cells were reacted with either H36-α_7 (E) or 05-α_7 (G) monoclonal antibody followed by fluorescein-conjugated donkey antimouse IgG, extracted with Triton X-100, and stained with anti-CD antibody and rhodamine-conjugated donkey anti-rabbit IgG (F) and (H). Laminin (10 μg/ml) (I) or 2E8 anti-laminin antibody (J) was added to cultures prior to fixation with ethanol and reaction with anti-CD antiserum and rhodamine-conjugated donkey anti-rabbit IgG. Bar, 20 μm.

Table 1. Crosslinking and ligand induced conformation change in the α_7 integrin chain

Primary antibody	Immunofluorescence	Association with cytoskeleton
H36-α_7	+++	yes
026-α_7	+++	yes
05-α_7	++	yes
A5	–	no
2E8 (anti-laminin)	+	yes

Conditions	Immunofluorescence	Association with cytoskeleton
20°C, 1% formaldehyde, 0.25% Triton X-100	–	no
20°C, 2% formaldehyde	–	no
20°C, 4% formaldehyde	–	no
37°C, 2% formaldehyde	–	no
20°C, 95% ethanol	–	no
37°C, 95% ethanol	–	no
45°C, 95% ethanol	++	yes
56°C, 95% ethanol	++	yes
20°C, 70% methanol,	++	yes
Laminin (1 μg/ml)	++	yes
Laminin (10 μg/ml)	+++	yes
Laminin (100 μg/ml)	+	yes

Detection of α_{7B} integrin with anti-cytoplasmic domain antiserum depends on crosslinking the integrin, temperature, or prior addition of laminin. For binding of the anti-cytoplasmic domain antiserum it was necessary to crosslink the α_{7B} chain with primary antibodies specific for the α_7 extracellular domain (H36-α_7, 026-α_7, 05-α_7) and then secondary antibody. The cells were extracted with Triton X-100, reacted with the rabbit anti-cytoplasmic domain antibody and fluorescein-conjugated donkey anti-rabbit IgG, and fixed with 95% ethanol. Antibody reactive with another molecule on the surface of these cells (A5) did not promote access to the cytoplasmic domain. Antibody reactive with laminin followed by secondary antibody, or laminin per se also promoted the changes in the cytoplasmic domain that affords access to the anti-cytoplasmic domain antiserum. Although fixation and permeabilization of the cells with 95% ethanol at room temperature or at 37°C did not allow binding of the anti-cytoplasmic domain antiserum, prior incubation at 45°C or 56°C did. The same conditions that promoted detection of the cytoplasmic domain also promoted the association of the integrin with the cytoskeleton and rendered it insoluble to extraction with Triton X-100.

and glycosylation at three potential sites in this peptide would decrease its mobility to approximately 38,000 Da (de Curtis et al., 1991; Parham et al., 1977). To demonstrate this, the integrin was treated with endoglycosidase F to remove carbohydrate. As a result, the mobility of the 38,000 Dapeptide changed to 26,000, confirming that one or more of the putative glycosylation sites inferred from amino acid sequence analysis (Song et al., 1992) is in fact used (Fig. 7B,C). The mobilities of the 121,000 and 70,000 Da polypeptides are also influenced by deglycosylation of the protein (Fig. 7B). Reactivity of the protein with the anti-cytoplasmic domain antiserum was inhibited by preincubation of the antibody with the peptide, confirming the indentity of the polypeptides reactive with the antibody (Fig. 7A).

In contrast with the results obtained by immunoblots, the results of immunofluorescence analyses of myoblasts and myotubes with this antiserum were negative or weak. In those experiments, the cells were treated either with 95% ethanol or 2% *p*-formaldehyde, both of which are routinely used to permeabilize these cells and render cytoplasmic proteins accessible to antibody. This failure of the cytoplasmic domain to react with the antiserum was apparently due to inaccessibility of the epitopes on the cytoplasmic domain, since immunoblots indicate that the α_7 chain is present subsequent to fixation.

Previous experiments demonstrated that reactivity of the α_7 chain with primary and secondary antibodies promotes the association of the integrin with the cell cytoskeleton, as noted by colocalization with actin filaments and a shift from being extractable in detergents such as Triton X-100 to becoming part of the detergent insoluble cytoskeletal network (Kaufman et al., 1985; Lowrey and Kaufman, 1989). The association of α_7 with the cytoskeleton upon crosslinking is rapid and dependent on bivalent secondary antibody. This association of α_7 with the cytoskeleton and change in its extractability suggested that upon crosslinking of the extracellular domain, the cytoplasmic domain may undergo a change in conformation or association with other proteins, and this might result in its accessibility to antibody. As seen in Fig. 8, reaction of α_7 integrin on live cells with monoclonal antibodies specific for the extracellular domain, followed by secondary antibody to crosslink the complexes, resulted in reactivity of the cytoplasmic domain with the antiserum. Antibodies reactive with other molecules in the membrane (A5, I3, H58 and H73; Kaufman and Foster, 1985) had no effect on accessibility of the α_{7B} cytoplasmic domain. Specific binding of the anti-cytoplasmic domain antibody was inhibited by prior incubation with the immunizing peptide. In these experiments the cells were first reacted with the monoclonal antibody followed by secondary donkey antimouse IgG. The cells were then either fixed using 95% ethanol or extracted with 0.25% Triton X-100 and then fixed. In both cases, staining with the antiserum was dependent on prior reactivity of cells with the primary and secondary antibodies, demonstrating that the accessibility of the cytoplasmic domain and shift in the capacity to extract α_7 with detergent were commensurate and dependent on crosslinking by the antibodies.

In addition to crosslinking α_7 in the membrane with antibody reactive with the α_7 chain, laminin promoted both the accessibility of the cytoplasmic domain to the anti-cytoplasmic domain antibody and the shift in α_7 extractability by detergent. The capacity of laminin to promote these changes was dose dependent and an excess of the extracellular matrix protein was suboptimal (Table 1) as would be expected if laminin too must bridge at least two α_7 molecules. Thus crosslinking this integrin, either with antibodies or its ligand, promotes its association with the cell cytoskeleton and an alteration in the cytoplasmic domain. Monoclonal antibody reactive with laminin could also promote this change to a limited extent. Exposure of the cells to 45°C or 56°C for 10 minutes, followed by fixation with 95% ethanol also rendered the cytoplasmic domain accessible to the antiserum, further indicating an unmasking of this region of the cytoplasmic domain by physical perturbations (Fig. 8; Table 1).

DISCUSSION

The $\alpha_7\beta_1$ integrin on skeletal myoblasts is a laminin-binding protein. First identified with monoclonal antibody H36 (Kaufman et al., 1985), expression of the α_7 integrin has

been used as a marker of the development of the skeletal myogenic lineages (Kaufman et al., 1991; George-Weinstein et al., 1993). The expression of α_7 during the development of the primary and secondary muscle fiber lineages differs and this is believed related to the diverse functions and requirement of these cells at different stages of development (George-Weinstein et al., 1993). During the development of primary muscle, α_7 is first seen on fibers, after terminal differentiation is initiated. This corresponds to the time these fibers envelop themselves in a laminin-rich extracellular matrix. It is within this lamina that secondary fiber formation subsequently takes place. In contrast with primary fiber formation, during the development of secondary fibers α_7 is expressed on precursor cells. The $\alpha_7\beta_1$ integrin may then serve to localize these precursor cells at the sites of secondary fiber formation and support the expansion of this population of cells. This is consistent with the findings that laminin selectively promotes a change in the shape and mobility of secondary myoblasts and maintains them proliferating (Foster et al., 1987; Ocalan et al., 1988). As $\alpha_7\beta_1$ is the sole functional laminin-binding integrin on skeletal myoblasts (von der Mark et al., 1991), we suggest that it has a significant role in mediating these behaviors of myoblasts in a laminin-rich environment.

Upon terminal differentiation, secondary myoblasts cease replicating, decrease their mobility, align and fuse to form multinulceate fibers. Immunofluorescence, immunoblots, and northern analyses demonstrate that an increase in the expression of α_7 integrin accompanies and is dependent on this stage of differentiation (Kaufman and Foster 1985; Kaufman et al., 1985; Song et al., 1992). Expression of the alternate A and C forms of the α_7 chain cytoplasmic domain takes place at this stage in development, as does the switch in expression of numerous other isoforms of muscle proteins, such as creatine kinase, NCAM, actin, and myosin.

Newly formed fibers associate laterally into bundles and distally with tendon. The α_7 integrin is localized along the membrane of mature muscle fibers (Song et al., 1992) and at myotendinous junctions (George-Weinstein and Kaufman, unpublished results), thereby participating in the distal and lateral cohesion of muscle fibers needed for the directed generation of force and movement. The roles of the α_7 integrin at these sites in functional muscle appear quite different from that in earlier myogenic development. We suggest that at least part of the functional diversity of the α_7 chain results from the use of alternate cytoplasmic domains.

The cytoplasmic domains of the 14 known alpha integrins have common as well as distinct features that undoubtedly underlie the function of these proteins. At the most amino terminal end of the cytoplasmic domains the alpha chains have a common sequence, GFFKR. This region may be essential for the stable association of α and β chains (Solowska et al., 1991). Some divergences in this region are in the GFFDR sequence of the chick $\alpha 8$ integrin (Bossy et al., 1991), the GFFNR sequence of the *Drosophila* PS2 chain (Bogaert et al., 1987), the DFFKP sequence of human α_{3B} (Takada et al., 1991) and as reported here, the GFFRR of α_{7A} and GFFKC of the α_{7C} form. The α_{3B}, α_{6B} and α_{7B} cytoplasmic domains have the common amino acid sequence, P-YHAV-I--E-R, which is highly homologous to a region in receptor-like protein tyrosine phosphatases. Whatever function this motif may serve is likely consistent in all these proteins. The α_3A, α_6A and α_{7A} cytoplasmic domains differ from the respective B forms in this region, but they are similar to each other, indicating that the functional changes between the A and B forms of α_3, α_6 and α_7 may be conserved. The remaining portions of these alpha chain isoforms and all other integrin α chain cytoplasmic domains are generally quite diverse from one another. Since the unique sequences of the cytoplasmic domains of the same integrin isoforms are highly conserved between species, it is reasonable to conclude that they are functionally significant. As demonstrated here, there is 84% identity in the human and rat α_{7B} cytoplasmic domains.

The potential roles of the alpha chain cytoplasmic domains are now coming under experimental scrutiny. Deletion or replacement of an alpha chain cytoplasmic domain can alter the binding affinity of the heterodimer to its ligand (O'Toole et al., 1991b) and alter its capacity to promote collagen gel contraction (Chan et al., 1992). These results imply that the cytoplasmic domain may confer specific functions to the integrins and perhaps participate in signal transduction, both into the cell and to the extracellular ligand-binding regions of the molecule. The expression of integrin alpha chain isoforms with different cytoplasmic domains at different stages of development (Cooper et al., 1991 and Tamura et al., 1991) further suggests that isoforms with different cytoplasmic domains may mediate specific functions during development. The developmentally regulated expression of multiple α_7 cytoplasmic domains supports this.

The mechanism of formation of the α_3, α_6, and α_7 A and B alternate cytoplasmic domains appears to be similar. The B forms originate from a splice site located 5′ to a second GFFKX coding region, that is downstream from the A form GFFXX coding regions (Cooper et al., 1991; Hogervorst et al., 1991; Tamura et al., 1991). In the case of α_{7A} and α_{7B} the 3′-splice site is followed by a common coding region in the A and B forms that is used in alternate reading frames. In contrast, the α_3 and α_6 3′-splice sites are in the 3′-untranslated regions and there is no common coding sequence in these A and B cytoplasmic domains. α_{7C} originates from an alternate splice that is common to a single GFFKX coding region and the 3′-untranslated region of α_{7B}. Identical splice site junctions, AAGTGTG, are found in α_{3B}, α_{6B} and α_{7C}, at the junctions of the transmembrane and cytoplasmic domains. The greatest homology between the α_{7B} integrin and other α chain isoforms is with α_{6B}: there is 47% identity in a 1,047 amino acid overlap, 70% identity in the transmembrane domains and 34% identity (and 77% homology) in a 56 amino acid overlap in the cytoplasmic domains. This suggests a common ancestry between the genes that encode these proteins, although the α_{7B} cytoplasmic domain appears to have evolved greater functional diversity.

Homologous phosphotransfer and ATP-binding sequences in the α_{7B} cytoplasmic domain suggest that it may have serine/threonine kinase activity, however, the catalytic activity of these kinases is localized in domains that are significantly larger than the 77 amino acids of the α_{7B} cytoplasmic domain. Alternatively, these motifs and the

phosphatase homology sequence in the α_{7B} cytoplasmic domain, may modulate the activity or localization of the respective enzymes, or other proteins that bind to them, at the inner periphery of the cell. The maintenance of the receptor-like protein tyrosine phosphatase homology region in α_{3B}, α_{6B} and α_{7B} suggests that whatever function it serves is conserved in the integrins that contain any of these alpha chains. In contrast, only the α_{7B} sequence contains the crucial lysine residue essential to serine/threonine protein kinase activity and both the phosphotransfer and ATP-binding motifs. It remains to be determined if α_{7B} has enzymatic activity, perhaps as a subunit of a larger complex, or if it has other regulatory functions.

The ATP-binding motif in the α_{7B} cytoplasmic domain contains and is adjacent to two additional DXHPX repeats. This threefold repeat is not evident in other sequences in current data banks. The 21 amino acid stretch that contains these repeats is highly hydrophobic and is 90% identical in human and rat.

Although we have not demonstrated that any of these motifs are functional within the α_{7B} cytoplasmic domain, they do present a rich potential for participating in the transduction of signals initiated outside the cell. Several interesting observations suggest that integrins are intimately involved in signal transductions in which phosphorylation and association with actin are certain to have key roles. The different potential tyrosine and serine/threonine phosphorylation sites in the three α_7 cytoplasmic domains could underlie diverse mechanisms of signal transduction at different stages of muscle development. The localization of integrin-regulated kinase activity ($pp125^{FAK}$) has recently been demonstrated at focal adhesions (Schaller et al., 1992), and tyrosine phosphorylation of $pp125^{FAK}$ appears to be activated by integrin and dependent on actin filaments (Lipfert et al., 1992). A single tyrosine residue in a protein kinase phosphorylation consensus site is present in all three α_7 cytoplasmic domains. Tyrosine phosphorylation of other cytoplasmic proteins also appears to be initiated through integrins and this may be enhanced in transformed cells. Phosphorylation of tyrosine in the integrin β_1 chain cytoplasmic domain in cells expressing $pp60^{v\text{-}src}$ leads to decreased association of the integrin with talin inside the cell and decreased association with extracellular matrix (Tapley et al., 1989; Horvath et al., 1990). As a consequence, these transformed cells become rounded. Modulation of the interactions of cells and extracellular matrix also take place during normal processes such as cell migration and cell division and these interactions too may be mediated by tyrosine phosphorylation (Hynes, 1992). The intimate association of α_7 and β_1 and the potential phosphotransfer activity, or kinase- and phosphatase-modulating capacities of the α_{7B} cytoplasmic domain, suggest that regulation of cell adhesion, mobility, shape and proliferation of myoblasts may take place through this integrin complex. In contrast with myogenic cells that exhibit normal expression of the α_7 integrin and differentiate, mutants that are deficient in α_7 have often lost their ability to control replication, are transformed and tumorigenic (Kaufman et al., 1985; Foster and Kaufman, 1985) and do not express α_{7A}. Association of extracellular matrix proteins with these cells is also altered. Thus both expression of α_{7B} and the switch to α_{7A} and α_{7C} appear to be important to myogenic differentiation. Immunologic reagents specific to the α_{7A} and α_{7C} forms will be used to confirm their expression and localization.

Immunolocalization of α_7 in the cell membrane and crosslinking α_7 integrin with antibodies reactive with its extracellular domain renders the α_{7B} cytoplasmic domain accessible to an antiserum raised against an immunogenic portion of its sequence. It should be noted that this crosslinking does not merely serve to aggregate these receptors and render them more readily detectable. The α_7 chain can be detected by immunofluorescence on cells processed at 4°C or fixed with 1% *p*-formaldehyde to prevent changes in its native distribution (Kaufman et al., 1985; Lowrey and Kaufman, 1989). Of more physiologic significance, incubation of cells with laminin also promotes unmasking within the cytoplasmic domain and this suggests that occupancy of the receptor in vivo also modulates such changes in the protein. It is highly likely that the capacity of laminin to promote the association between integrin and the cell cytoskeleton is significant to the changes in mobility, shape and proliferative state of these cells growing in a laminin environment. The actin-binding motif in the α_{7B} cytoplasmic domain is similar to that in villin (Friederich et al., 1992) and may be directly involved in this association. The change in reactivity of the cytoplasmic domain with antibody promoted by antibody or ligand may result from altering the association of the α_7 cytoplasmic domain with other proteins, and/or, from a conformational change initiated in the extracellular portion of the molecule. In either case, modulation of the structure of the integrin cytoplasmic domain initiated outside the cell likely results in the physiologic responses of these cells. Similarly, activation of the platelet $\alpha_{IIb}\beta_3$ integrin by thrombin or collagen, or by antibodies reactive with the receptor, leads to a change in conformation in the β chain that also results in its accessibility to an antibody (Shatill et al., 1985; Gulino et al., 1990; Kouns et al., 1990; Andrieux et al., 1991; O'Toole et al., 1991a). Fab' fragments of secondary antibodies are ineffective at promoting association of α_7 with the cytoskeleton, indicating that bridging at least two $\alpha_7\beta_1$ heterodimers is necessary to alter its cytoplasmic domain and direct its interaction with the cell cytoskeleton (Lowrey and Kaufman, 1989). This suggests that some signals are transduced into the cell by a mechanism in which integrin crosslinking and association with the cytoskeleton are important factors. As discussed, the α_{7B} cytoplasmic domain does contain a rich potential for participating in the transduction of signals initiated outside the cell. Further defining the roles of the motifs in the α_{7B} cytoplasmic domain and the diversity in functions that arise from use of the alternate cytoplasmic domains is of great interest to understanding the significance of extracellular matrix and integrins in the development of skeletal muscle, and the mechanisms and molecules involved in matrix induced signal transduction.

We thank Mr Maojian Gu for his skillful assistance with the endoglycosidase experiments and Dr George Dickson for generously providing the human fetal muscle cDNA library. We also thank Dr Vito Quaranta for allowing us to discuss results from

his laboratory prior to their publication. This work was supported by National Institutes of Health grant GM28842. The α_{7A}, α_{7B} and α_{7C} nucleotide sequence data are available from the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession numbers X74293, X74295 and X74294, respectively.

REFERENCES

Aitken, A. (1990). *Identification of Protein Consensus Sequences: Active Site Motifs, Phosphorylation, and other Post-translational Modifications*, pp. 152-160. Ellis Horwood, New York.

Albelda, S. M. and Buck, C. A. (1990). Integrins and other cell adhesion molecules. FASEB J. 4, 2868-2880.

Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G. and Silengo, L. (1990). A human integrin β_1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene* 95, 261-266.

Andrieux, A., Rabiet, M. J., Chapel, A., Concord, E. and Marguerie, G. (1991). A highly conserved sequence of the Arg-Gly-Asp-binding domain of the integrin β_3 subunit is sensitive to stimulation. *J. Biol. Chem.* **266**, 14202-14207.

Altschul, S. F., Gish, W., Miller W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Bogaert, T., Brown, N. and Wilcox, M. (1987). The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell* **51**, 929-940.

Bossy, B., Bossy-Wetzel, E. and Reichardt, L. F. (1991). Characterization of the $\alpha 8$ subunit: a new integrin β_1-associated subunit, which is prominently expressed on axons and on cells in contact with basal laminae in chick embryos. *EMBO J.* **10**, 2375-2385.

Chan, B. M. C., Kassner, P. D., Schiro, J. A., Byers, R., Kupper, T. S. and Hemler, M. E. (1992). Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell* **68**, 1051-1060.

Chou, P. Y. and Fasman, G. D. (1974). Conformational parameters for amino acids in helical, beta-sheet, and random coil regions calculated from proteins. *Biochemistry* **13**, 211-222

Collo, G., Starr, L. and Quaranta, V. (1993) A new isoform of the laminin receptor integrin $\alpha_7\beta_1$ is developmentally regulated in skeletal muscle. *J. Biol. Chem.* (in press).

Cooper, H. M., Tamura, R. N. and Quaranta, V. (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the $\alpha_6\beta_1$ integrin. *J. Cell Biol.* **115**, 843-850.

Dahl, S. C. and Grabel, L. B. (1989). Integrin phosphorylation is modulated during the differentiation of F-9 teratocarcinoma stem cells. *J. Cell Biol.* **108**, 183-190.

de Curtis, I., Quaranta, V., Tamura, R. N. and Reichardt, L. F. (1991). Laminin receptors in the retina: sequence analysis of the chick integrin α_6 subunit. *J. Cell Biol.* **113**, 405-416.

Engvall, E., Davis, G. E., Dickerson, K., Rouslahti, E., Varon, S. and Manthorpe, M. (1986). Mapping of domains in human laminin using monoclonal antibodies: localization of the neurite-promoting site. *J. Cell Biol.* **103**, 2457-2465.

Foster, R. F. and Kaufman, S. J. (1985). Cell-surface events during myogenesis: immunofluorescence analysis using monoclonal antibodies. In *Investigation and Exploitation of Antibody Combining Sites* (ed. E. Reid, G. M. W. Cook and D. J. Morre) pp. 167-176. Plenum, New York.

Foster, R. F., Thompson, J. M. and Kaufman, S. J. (1987). A laminin substrate promotes myogenesis in rat skeletal muscle cultures: analysis of replication and development using antidesmin and anti-BrdUrd monoclonal antibodies. *Dev. Biol.* **122**, 11-20.

Friederich, E., Vancompernolle, K., Huet, C., Goethals, M., Finidori, J., Vandekerckhove, J. and Louvard, D. (1992). An actin-binding site containing a conserved motif of charged amino acid residues is essential for the morphogenic effect of villin. *Cell* **70**, 81-92.

Garnier, J., Osguthorpe, D. J. and Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97-120.

Gebbink, M. F. B. G., van Etten, I., Hateboer, G., Suijkerbuijk, R., Beijersbergen, R. L., van Kessel, A. G. and Moolenaar, W. H. (1991). Cloning, expression and chromosomal localization of a new putative receptor-like protein tyrosine phosphatase. *FEBS Lett.* **290**, 123-130.

George-Weinstein, M., Foster, R. F., Gerhart, J. V. and Kaufman, S. J. (1993). In vitro and in vivo expression of α_7 integrin and desmin define the primary and secondary myogenic lineages. *Dev. Biol.* **156**, 209-229.

Goodman, S. L., Risse, G. and von der Mark, K. (1989). The E8 subfragment of laminin promotes locomotion of myoblasts over extracellular matrix. *J. Cell Biol.* **109**, 799-809.

Gulino, D., Ryckewaert, J. J., Andrieux, A., Rabiet, M. J. and Marguerie, G. (1990). Identification of a monoclonal antibody against platelet GPIIb that interacts with a calcium-binding site and induces aggregation. *J. Biol. Chem.* **265**, 9575-9581.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1986). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Hemler, M. E. (1990). VLA proteins in the integrin family: structure, functions and their roles on leukocytes. *Annu. Rev. Immunol.* **8**, 365-400.

Hirst, R., Horwitz, A., Buck, C. and Rohrschneider, L. (1986). Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Nat. Acad. Sci. USA* **83**, 6470-6474.

Holton, T. A. and Graham, M. W. (1990). A simple and sufficient method for direct cloning of PCR products using ddT-tailed vector. *Nucl. Acids Res.* **19**, 1156.

Hopp, T. P. and Woods, K. R. (1981). Predication of protein antigenic determinants from amino acid sequences. *Proc. Nat. Acad. Sci. USA* **78**, 3824-3828.

Hogervorst, F., Kuikman, I., van Kessel, A. G. and Sonnenberg, A. (1991). Molecular cloning of the human α_6 integrin subunit. Alternative splicing of α_6 mRNA and chromosomal localization of the α_6 and $\beta 4$ genes. *Eur. J. Biochem.* **199**, 425-433.

Horvath, A. R., Elmore, M. A. and Kellie, S. (1990). Differential tyrosine-specific phosphorylation of integrin in Rous sarcoma virus transformed cells with differing transformed phenotypes. *Oncogene* **5**, 1349-1357.

Horwitz, A., Duggan, K., Buck, C., Beckerle, K. and Burridge, K. (1986). Interaction of plasma membrane fibronectin with talin: a transmembrane linkage. *Nature* **320**, 531-533.

Hynes, R. O.(1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Juliano, R. L. and Haskill, S. (1993). Signal transduction from the extracellular matrix. *J. Cell Biol.* **120**, 577-585.

Kaufman, S. J. and Foster, R. F. (1985). Remodeling of the myoblast membrane accompanies development. *Dev. Biol.* **110**, 1-14.

Kaufman, S. J. and Foster, R. F. (1988). Replicating myoblasts express a muscle-specific phenotype. *Proc. Nat. Acad. Sci. USA.* **85**, 9606-9610.

Kaufman, S. J., Foster, R. F., Haye, K. R. and Faiman, L. E. (1985). Expression of a developmentally regulated antigen on the surface of skeletal and cardiac muscle cells. *J. Cell Biol.* **100**, 1977-1987.

Kaufman, S. J., George-Weinstein, M. and Foster, R. F. (1991). In vitro development of precursor cells in the myogenic lineage. *Dev. Biol.* **146**, 228-238.

Kaufman, S. J. and Parks, C. M. (1977). Loss of growth control and differentiation in the fu-1 variant of the L8 line of rat myoblasts. *Proc. Nat. Acad. Sci. USA* **74**, 3888-3892.

Kouns, W. C., Wall, C. D., White, M. M., Fox, C. F. and Jennings, L. K. (1990). A conformation-dependent epitope of human platelet glycoprotein IIIa. *J. Biol. Chem.* **265**, 20594-20601.

Kramer, R. H., Vu, M. P., Cheng, Y.-F., Ramos, D. M., Timpl, R. and Waleh, N. (1991). Laminin-binding integrin $\alpha_7\beta_1$: functional characterization and expression in normal and malignant melanocytes. *Cell Regul.* **2**, 805-817.

Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J.T. and Brugge, J. S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J. Cell Biol.* **119**, 905-912.

Lowrey, A. A. and Kaufman, S. J. (1989). Membrane-cytoskeleton associations during myogenesis deviate from traditional definitions. *Exp. Cell Res.* **183**, 1-23.

Ocalan, M., Goodman, S. L., Kuhl, U., Hauschka, S. D. and von der Mark, K. (1988). Laminin alters cell shape and stimulates motility and proliferation of murine skeletal myoblasts. *Dev. Biol.* **125**, 158-167.

O'Toole, T. E., Loftus, J. C., Du, X. P., Glass, A. A., Ruggeri, Z. M., Shattil, S. J., Plow, E. F. and Ginsberg, M. H. (1991a). Affinity modulation of the $\alpha IIb\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regul.* **1**, 883-893.

O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F. and

Ginsberg, M. H. (1991b). Modulation of the affinity of integrin $\alpha IIb\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of αIIb. *Science* **25**, 845-847.

Otey, C. A., Pavalko, F. M. and Burridge, K. (1990). An interaction between α-actinin and the β_1 integrin subunit in vitro. *J. Cell Biol.* **111**, 721-729.

Parham, P., Alpert, B. N., Orr, H. T., and Strominger, J. L. (1977). Carbohydrate moiety of HLA antigens. Antigenic properties and amino acid sequences around the site of glycosylation. *J. Biol Chem.* **252**, 7555-7567.

Reszka, A. A., Hayashi, Y. and Horwitz, A. F. (1992). Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* **117**, 1321-1330.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992). pp125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Nat. Acad. Sci. USA* **89**, 5192-5196.

Silhavy, T. J., Berman, M. L. and Enquist, L. W. (1984). Rapid isolation of λ DNA. *Experiments with Gene Fusions*, pp. 142-143. Cold Spring Harbor Laboratory Press, New York.

Shattil, S. J., Hoxie, J. A., Cunningham, M. and Brass, L. F. (1985). Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J. Biol. Chem.* **260**, 11107-11114.

Solowska, J., Edelman, J. M., Albelda, S. M. and Buck, C. A. (1991). Cytoplasmic and transmembrane domains of integrin β_1 and β_3 subunits are functionally interchangeable. *J. Cell Biol.* **114**, 1079-1088.

Song, W. K., Wang, W., Foster, R. F., Bielser, D. A. and Kaufman, S. J. (1992). H36-α_7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* **117**, 643-657.

Springer, T. A. (1990). The sensation and regulation of interactions with extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annu. Rev. Cell Biol.* **6**, 359-402.

Springer, S. J. and Paradiso, P. R. (1981). A transmembrane link between fibronectin and vinculin (130-kd protein): serum modulation in normal and transformed hamster fibroblasts. *Cell* **24**, 481-492.

Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M. H. and Hemler, M. E. (1991). Molecular cloning and expression of cDNA for the alpha 3 subunit of human alpha 3 beta 1 (VAL-3), an integrin receptor for fibronectin, laminin and collagen, *J. Cell Biol.* **115**, 257-266.

Tamura, R. N., Cooper, H. M., Collo, G. and Quaranta, V. (1991). Cell type-specific integrin variants with alternative α chain cytoplasmic domains. *Proc. Nat. Acad. Sci. USA* **88**, 10183-10187.

Tamura, R. N., Rozzo, C., Starr, L., Chamber, J., Reichardt, L. F., Cooper, H. M. and Quaranta, V. (1990). Epithelial integrin $\alpha_6\beta 4$: complete primary structure of α_6 and variant forms of β4. *J. Cell Biol.* **111**, 1593-1604.

Tapley, P., Horwitz, A. F., Buck, C. A., Burridge, K., Duggan, K., Hirst, R. and Rohrschneider, L. (1989). Analysis of the avian fibronectin receptor (integrin) as direct substrate for $pp60^{v\text{-}src}$. *Oncogene* **4**, 325-333.

van Kuppevelt, T. H. M. S. M., Languino, L. R., Gailit, J. O., Suzuki, S. and Ruoslahti, E. (1989). An alternative cytoplasmic domain of the integrin β_3 subunit. *Proc. Nat. Acad. Sci. USA* **86**, 5415-5418.

von der Mark, H., Durr, J., Sonnenberg, A., von der Mark, K., Deutzmann, R. and Goodman, S. L. (1991). Skeletal muscle utilize a novel β_1-series integrin and not $\alpha_6\beta_1$ for binding to the E8 and T8 fragments of laminin. *J. Biol. Chem.* **266**, 23593-23601.

(Received 7 June 1993 - Accepted 18 August 1993)

APPENDIX 4

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Identification of Multiple Cell Adhesion Receptors for Collagen and Fibronectin in Human Fibrosarcoma Cells Possessing Unique α and Common β Subunits

Elizabeth A. Wayner* and William G. Carter*‡

* Department of Biochemical Oncology, Fred Hutchinson Cancer Research Center and Pacific Northwest Research Foundation; and ‡ Department of Pathobiology, University of Washington, Seattle, Washington 98104

Abstract. Using monoclonal antibody technology and affinity chromatography we have identified four distinct classes of cell surface receptors for native collagen on a cultured human fibrosarcoma cell line, HT-1080. Two classes of monoclonal antibodies prepared against HT-1080 cells inhibited adhesion to extracellular matrix components. Class I antibodies inhibited cell adhesion to collagen, fibronectin, and laminin. These antibodies immunoprecipitated two noncovalently linked proteins (subunits) with molecular masses of 147 and 125 kD, termed α and β, respectively. Class II antibodies inhibited cell adhesion to native collagen only and not fibronectin or laminin. Class II antibodies immunoprecipitated a single cell surface protein containing two noncovalently linked subunits with molecular masses of 145 and 125 kD, termed α and β, respectively. The two classes of antibodies did not cross-react with the same cell surface protein and recognized epitopes present on the α subunits. Pulse-chase labeling studies with [^{35}S]methionine indicated that neither class I nor II antigen was a metabolic precursor of the other. Comparison of the α and β subunits of the class I and II antigens by peptide mapping indicated that the β subunits were identical while the α subunits were distinct. In affinity chromatography experiments HT-1080 cells were extracted with Triton X-100 or octylglucoside detergents and chromatographed on insoluble fibronectin or native type I or VI collagens. A single membrane protein with the biochemical characteristics of the class I antigen was isolated on fibronectin-Sepharose and could be immunoprecipitated with the class I monoclonal antibody. The class I antigen also specifically bound to type I and VI collagens, consistent with the observation that the class I antibodies inhibit cell adhesion to types VI and I collagen and fibronectin. The class II antigen, however, did not bind to collagen (or fibronectin) even though class II monoclonal antibodies completely inhibited adhesion of HT-1080 cells to types I and III-VI collagen. The class Iβ and IIβ subunits were structurally related to the β subunit of the fibronectin receptor described by others. However, none of these receptors shared the same α subunits. Additional membrane glycoprotein(s) with molecular mass ranges of 80–90 and 35–45 kD, termed the class III and IV receptors, respectively, bound to types I and VI collagen but not to fibronectin. Monoclonal antibodies prepared against the class III receptor had no consistent effect on cell attachment or spreading, suggesting that it is not directly involved in adhesion to collagen-coated substrates.

These results suggest that the class I and II receptors are two new members of a family of cell surface receptors for the extracellular matrix involved in mediating cell adhesion and shall be referred to as ECMRI and II. Each member of this family possesses a common β subunit and a unique α subunit. The class II receptor appears to be a primary mediator of specific cell adhesion to collagen. The promiscuous class I receptor also mediates cell adhesion to collagen but appears to interact with fibronectin and laminin as well. The class III receptor is not a member of this family of extracellular matrix adhesion receptors but does bind specifically to native collagens.

FIBRONECTIN has been shown to interact with a family or families of related cell surface proteins with molecular masses of ~140 kD in fibroblasts (Brown and Juliano, 1985, 1986; Akiyama et al., 1986; Wylie et al., 1979), osteosarcoma cells (Pytela et al., 1985), endothelial cells (Plow et al., 1986), lymphoid cells (Brown and Juliano, 1986), platelets (Pytela et al., 1986; Gardner and Hynes, 1985), and muscle cells (Horwitz et al., 1985; Dambsky et al., 1985; Chapman, 1984). The interaction of fibronectin with this cell surface receptor appears to mediate cell adhesion to fibronectin and to proceed via interaction of the receptor with the amino acid sequence RGDS located in the

cell attachment domain of fibronectin (Pierschbacher and Ruoslahti, 1984*a*, *b*; Akiyama et al., 1985).

However, the extracellular matrix (ECM)[1] of untransformed cultured human fibroblasts, such as WI-38 cells, contains large quantities of at least two other transformation-sensitive adhesion proteins, GP250 and GP140 (Carter and Hakamori, 1981; Carter, 1982*a*, *b*), as well as fibronectin. GP140 is a hybrid protein with a major globular domain and a minor collagenous domain that corresponds to the α2 and/or α1 subunits of type VI collagen isolated from pepsin digests of human placenta (Heller-Harrison and Carter, 1984). Purified, nonreduced, and nonpepsinized type VI collagen, or GP140, as well as GP250, can induce stable cell adhesion and spreading; demonstrating that the ECM contains major adhesive proteins other than fibronectin. Since the ECM is therefore a complex organization of adhesive proteins (and other macromolecular substances such as glycosaminoglycans) it is reasonable to hypothesize the existence of cell surface receptors other than the fibronectin receptor for ECM adhesive components, such as type VI or other collagens. In fact, Hayman et al. (1985) have shown that cell adhesion to collagen is not inhibited by RGDS-containing peptides. Similarly, Nagata et al. (1985) concluded that collagen noncompetitively inhibited cell spreading but not cell attachment to fibronectin. These data strongly suggest that cell adhesion to collagen is mediated by a receptor distinct from the fibronectin receptor. Despite considerable effort, conclusive evidence implicating a particular cell surface receptor(s) in attachment to collagen has not been presented even though various cell surface proteins that do interact with collagen have been described in platelets (Chiang and Kang, 1982; Kotite and Cunningham, 1986; Santoro, 1986; Saito et al., 1986), chondrocytes (Mollenhauer and von der Mark, 1983; Mollenhauer et al., 1984), hepatocytes (Rubin et al., 1981), and other cells (Kurkinen et al., 1984).

Furthermore, since type VI collagen differs from collagen types I–V in various ways, there is also the possibility that the cell surface receptor involved in mediating cell adhesion or assembly of type VI collagen into the ECM may be different than the receptor(s) for other collagen types. The unique characteristics of type VI collagen relative to other collagen types include: (*a*) a relatively low affinity for the gelatin-binding domain of plasma fibronectin when compared with collagen types I–V (Carter, 1982*b*; Heller-Harrison, R. A. and W. G. Carter, manuscript in preparation), (*b*) extensive intermolecular disulfide bonding and complex-type glycosylation within the pepsin-resistant domains (Heller-Harrison and Carter, 1984; Carter, 1984), (*c*) resistance to digestion with bacterial collagenase in the nonreduced state (Heller-Harrison and Carter, 1984), and (*d*) preferential accumulation in the ECM as a detergent-insoluble complex (half-life of 73 h) in contrast to other collagens (half-life of 5 h) that accumulate as soluble components in cell culture media (Carter, 1982*a*).

In preliminary work we described three cell surface glycoproteins with molecular masses of 140, 80–90, and 45 kD that bound to type VI collagen in affinity chromatography experiments and considered to be potential cell surface receptors for type VI collagen involved in cell adhesion (Carter and Wayner-Carter, 1986). In the present paper we have extended the previous studies to include inhibition of cell attachment with monoclonal antibodies as well as affinity chromatography on insolubilized ECM components in order to define four cell surface proteins (classes I–IV) with affinity for type VI collagen (and/or fibronectin). We also present conclusive evidence directly implicating class I and II receptors in the collagen adhesion process. The role of these receptors in mediating cell adhesion to type VI and other collagen types as well as fibronectin is examined and discussed.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FNR, fibronectin receptor; 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide.

Materials and Methods

Materials

Phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), diisopropyl fluorophosphate, 2-mercaptoethanol (2-ME), BSA, Triton X-100, *n*-octyl-β-D-glucopyranoside, EDTA, thermolysin (protease type X), pepsin, protein A–agarose, gelatin (swine skin), tuftsin peptide, and V8 protease (from *Staphylococcus aureus*, strain V8, protease type XVII) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactoperoxidase and glucose oxidase were from Calbiochem-Behring Corp. (San Diego, CA). Fluorescein-conjugated (goat) anti-mouse IgG and IgM (H and L chains) were obtained from Tago, Inc. (Burlingame, CA). Rabbit anti-mouse IgG (H and L) antiserum was obtained from Cappel (CooperBiomedical, Malvern, PA). [^{51}Cr]Sodium chromate was from New England Nuclear. [^{35}S]methionine (Trans ^{35}S-label, 1010 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). ^{125}I was from Amersham Corp. (Arlington Heights, IL). The GRGDS peptide was obtained from Peninsula Laboratories Inc. (Belmont, CA). Rabbit polyclonal antibodies prepared against the fibronectin receptor as described by Pytela et al. (1985) were the generous gift of Dr. M. D. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA).

Cells and Cell Culture

Normal embryonic human lung diploid fibroblasts, WI-38 cells, and SV-40 virus transformants of WI-38 cells, WI-38 VA13 cells, a human fibrosarcoma cell line, HT-1080 cells, and a human rhabdomyosarcoma cell line, A204 cells, were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium and 10% FBS in a 5% CO_2 atmosphere.

Preparation of Monoclonal Antibodies

Monoclonal antibodies were produced by the methods of Oi and Herzenberg (1980) and Taggart and Samloff (1983). Briefly, either BALB/c or RBF/Dn mice (Jackson Laboratory, Bar Harbour, ME) were immunized with nontrypsinized WI-38 VA13 or HT-1080 cells. Immune spleens were removed and fused with either SP2/0, or the NS-1/FOX-NY myeloma cell lines. Viable heterokaryons were selected in RPMI 1640 supplemented with hypoxanthine/aminopterin/thymidine or adenine/aminopterine/thymidine. Heterokaryons were screened for the production of antibodies in a three-stage assay as described below. After screening, selected heterokaryons were cloned by limiting dilution with thioglycolate-induced irradiated BALB/c peritoneal macrophages or thymocytes as feeder cells.

Screening of Heterokaryons

Stage One: Solid-Phase Assay. Heterokaryons that produced antibodies that reacted with HT-1080 cells were identified in a solid-phase assay. HT-1080 cells were grown to confluence in 96-well culture plates (No. 3072; Falcon Labware, Oxnard, CA), washed with PBS, fixed with 4% wt/vol paraformaldehyde in PBS, blocked with 10% FBS, and then reacted with culture supernatant from the heterokaryons. The HT-1080 cells were washed, reacted with the secondary antibody rabbit anti-mouse IgG (H and L chains), followed by reaction with ^{125}I-protein A and autoradiography of the culture plates.

Stage Two: Immune Precipitation. Heterokaryons producing antibodies to cell surface proteins of HT-1080 cells were identified by immunoprecipitation. HT-1080 cells were surface-labeled with radioactive iodine using the lactoperoxidase/glucose oxidase method (Hynes, 1973), followed by extraction with 1% vol/vol Triton X-100 detergent in 25 mM Tris-HCl buffer (Tri-

ton/Tris), pH 7.5, containing 1 mM diisopropyl fluorophosphate or 1 mM PMSF plus 2 mM NEM as protease inhibitors. After centrifugation at 35,000 *g* for 20 min the soluble detergent extract was mixed with BSA coupled to Sepharose (BSA-Sepharose) and then recentrifuged to preclear the labeled extract. The extract was immunoprecipitated with cell culture supernatants from the heterokaryons. To reduce the number of samples to be precipitated, the culture supernatants were placed in a matrix organization and then pooled in columns and rows so every supernatant was present in two pools derived from one column and one row. The pooled supernatants were then used for immunoprecipitation. The precipitates from the columns and rows could then be used to identify supernatants with specific antigen reactivity. Immunoprecipitation proceeded as follows. (*a*) Rabbit anti-mouse IgG was bound to protein A-agarose by incubation in Triton/Tris containing 0.5% wt/vol BSA (Triton/Tris/BSA) and then washed by centrifugation. (*b*) The immobilized secondary antibodies were incubated with the pooled heterokaryon culture supernatants to immobilize the primary antibodies, then washed by centrifugation. (*c*) The immobilized primary antibodies were incubated with the labeled HT-1080 cell extract, washed in Triton/Tris containing 400 mM NaCl, and the bound antigens visualized on SDS-PAGE gels by autoradiography.

Stage Three: Inhibition of Cell Adhesion. Antibodies that would alter cell adhesion to either or both fibronectin and type VI collagen were selected as follows. (*a*) 48-well virgin styrene plates were coated with pepsin-generated type VI collagen or human plasma fibronectin (200 μl protein solution/well, 5 μg protein/ml) as previously described (Rauvala et al., 1981; Carter, 1982*a*). The plates were blocked with 200 μl PBS supplemented with 10 mg/ml heat-denatured BSA (80°C for 3 min). The 48-well plates were obtained by special order from Costar (Van Nuys, CA) and consisted of a culture plate (No. 3548, Costar) that had not been treated to induce cell adhesion. In the absence of exogenous adhesive protein, HT-1080 cells would not adhere to these plates. (*b*) HT-1080 cells were labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml for 2–4 h; New England Nuclear, Boston, MA) and were suspended with trypsin (0.05% wt/vol) in PBS containing 5 mM EDTA as previously described (Carter, 1982*a*). The cells (2×10^5/well) were incubated with the heterokaryon culture supernatants (1:2 dilution in PBS supplemented with 1 mg/ml heat-denatured BSA) or control SP2 cell culture supernatant for 1 h at 4°C with agitation, then the cells were allowed to adhere to the fibronectin- or collagen-coated surfaces in the presence of the hybridoma supernatants for 15–30 min at room temperature or 37°C. (*c*) Nonadherent cells were removed by washing with PBS. The adherent cells were dissolved in SDS/NaOH and quantitated in a gamma counter.

Sequential Immune Precipitation

Detergent extracts from HT-1080 cells labeled with radioactive iodine were subjected to three cycles of immunoprecipitation using antibodies bound to protein A-agarose. Each cycle of precipitation involved the following steps: (*a*) incubation of the antibody-agarose with the labeled cell extract; (*b*) separation of the antigen antibody agarose from the unbound extract by centrifugation; and (*c*) incubation of the next antibody-agarose complex with the unbound extract from step *b*.

Preparation of Adhesive Proteins

Fibronectin. Human plasma fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose according to the method of Engvall and Ruoslahti (1977).

Cell Adhesion Domain of Fibronectin. Human plasma fibronectin was digested with thermolysin as described by Sekiguchi and Hakomori (1983) and the gelatin-binding domain was removed by affinity chromatography on gelatin-Sepharose. The 140-kD cell adhesion domain was isolated by chromatography on Sephacryl S-200.

Nonpepsinized Type VI Collagen. Type VI collagen was isolated from human placenta without pepsin digestion or reduction of disulfide bonds by extraction of urea-insoluble residue with sodium trichloroacetate (NaTCA) as previously described (Carter, 1982*a*). Briefly, human placentae were washed in PBS to remove blood and debris, and were then homogenized in PBS in a blender (Waring Products, New Hartford, CT). All steps were carried out in the presence of 1 mM PMSF, 2 mM NEM, and 5 mM EDTA. The homogenate was centrifuged at 25,000 *g* for 15 min and pellets were sequentially extracted with 25 mM Tris-HCL, pH 7.8, containing 2% Empigen BB and 0.1% SDS; and twice with 25 mM Tris-HCl, pH 7.8, containing 8 M urea with 1 M NaCl. The resulting pellet was homogenized in 4 M NaTCA and incubated with stirring for 4 h. After centrifugation, the supernatant fraction, which contained most of the type VI collagen, was dialyzed extensively against water. During dialysis the material which precipitated was collected by centrifugation, redissolved in 8 M urea in 25 mM Tris-HCL, pH 7.8, and finally dialyzed against 0.5 M acetic acid. The dialysate was centrifuged, and the supernatant fraction containing the interstitial collagens and small quantities of types IV and V collagens was discarded. Trace quantities of other collagens were removed by digestion with bacterial collagenase under nonreducing conditions. In the absence of reducing agents type VI collagen is resistant to digestion with collagenase. The type VI collagen was further purified by molecular sieve chromatography in 8 M urea or SDS on Sephacryl S500.

Pepsinized Collagen Types I and III–VI. All collagen types were isolated from fresh human placenta after digestion with pepsin as described by Miller and Rhodes (1982) for collagen types I and III–V, and as described by Jander et al. (1981) for collagen type VI.

Laminin. Mouse laminin was isolated from Englebreth-Holm-Swarm sarcoma grown in BALB/c mice as described by Timpl et al. (1979).

Pulse-Chase Experiments

HT-1080 cells were grown to confluence in 6.0-cm culture plates (1×10^6 cells/plate), washed with methionine-free culture medium, and incubated in the same medium for 2 h. The methionine-starved cells were pulse-labeled with [^{35}S]methionine (75 μCi/ml, 1.5 ml/plate) for 1 h, and then washed and chased with RPMI medium containing 10% FBS. At the indicated time points the plates were washed with PBS, 1.0 ml of Triton/Tris buffer containing 1 mM PMSF was added to each plate, and the cell residue scraped with a rubber policeman. The detergent suspension was incubated on ice for 30 min, centrifuged at 35,000 *g* for 20 min and 100-μl aliquots of the supernatant quantitatively immunoprecipitated with the indicated antibodies as described above. The immunoprecipitates were visualized on SDS-PAGE gels by fluorography using Enlightning (Bio-Rad Laboratories, Richmond, CA).

Peptide Analysis

Peptide analysis followed the basic procedure of Cleveland et al. (1977) with modifications as follow. (*a*) Class I and II antigens were immunoprecipitated from detergent extracts of iodine-labeled HT-1080 cells. (*b*) The precipitated antigens were fractionated on SDS-PAGE gels (7% acrylamide) in the absence of 2-ME in order to resolve the α and β subunits of both antigens. The gels were dried, without fixation, and then visualized by autoradiography in order to locate the labeled subunits. (*c*) The dried regions of the gels containing the α and β subunits of the class I and II antigens were excised and placed in the wells of a second SDS-PAGE gel (20% acrylamide) and allowed to rehydrate in the presence of sample buffer for 1 h. (*d*) V8 protease in sample buffer was added to each well (500 ng protease/sample) of the gel and incubated for 60 min at room temperature. The completed gels were visualized by autoradiography.

Polyacrylamide Gel Electrophoresis

SDS-PAGE gels were prepared following the basic stacking gel system of Laemmli (1970). Samples prepared under reducing conditions were dissolved in a sample buffer containing 2% wt/vol SDS with 5% vol/vol 2-ME and heated in a boiling water bath for 5 min. Samples prepared under nonreducing conditions were treated as above except that 10 mM NEM was substituted for the 2-ME. Prestained protein standards for relative molecular mass estimation were obtained from Bethesda Research Laboratories (Bethesda, MD) and were as follows: lysozyme, 14,300; B-lactoglobulin, 18,400; A-chymotrypsin, 25,700; ovalbumin, 43,000; BSA, 68,000, phosphorylase B, 97,400; and myosin (H chain) 200,000. Protein was determined by the fluorescamine method (Udenfriend et al., 1972).

Preparation of Affinity Resins

Human plasma fibronectin, purified collagen types I and VI, BSA, and gelatin were coupled to Sepharose CL-4B using the cyanogen bromide activation method (Parikh et al., 1974).

Differential Cell Extracts

HT-1080 cells were surface labeled following the lactoperoxidase method and ^{125}I (Hynes, 1973). Labeled cells were extracted sequentially in order to prepare subpopulations of labeled cell proteins. The sequential extraction protocol was basically as previously described (Carter, 1982*b*) and involved

homogenization of labeled cells with a glass Dounce homogenizer (type A) in 25 mM Tris-HCl buffer, pH 7.5, and 1 mM PMSF at 4°C plus the following in sequence: (*a*) 0.34 M sucrose and 1 mM EDTA, in the absence of detergent to solubilize cytoplasmic and peripherally associated cell surface components; (*b*) 1% vol/vol Triton X-100 detergent to solubilize components that required detergent for solubilization; and (*c*) 0.5% wt/vol SDS at 100°C for 5 min to solubilize residual material.

Affinity Chromatography

Cell extracts were chromatographed on BSA-Sepharose in detergent/Tris buffer containing 0.5% wt/vol BSA (detergent/Tris/BSA) to preclear the extracts. Equal aliquots of the labeled proteins were chromatographed on various affinity columns (10 ml protein–Sepharose/column) in the detergent/Tris/BSA buffer. The columns were washed with 2 bed vol of detergent/Tris/BSA, 2 bed vol of detergent/Tris, and 5 bed vol of 0.1% vol/vol detergent/Tris to remove unbound proteins. The labeled proteins bound to the affinity columns were eluted with final wash buffer containing 200 mM NaCl and/or 6 M urea as indicated. The eluted components were dialyzed against water to remove NaCl and urea, lyophilized, and redissolved in water.

Fibronectin receptor was isolated from octylglucoside extracts basically as described by Pytela et al. (1985) and as follows. Labeled HT-1080 cells were extracted in 100 mM octylglucoside detergent in PBS at 4°C containing 1 mM PMSF as a protease inhibitor. The octyglucoside extracts were chromatographed on insoluble fibronectin at 4°C and washed with octylglucoside (25 mM) in PBS. Tuftsin peptide (thr-lys-pro-arg, 1 mg/ml) failed to elute the fibronectin receptor while the peptide gly-arg-gly-asp-ser (1 mg/ml) specifically eluted a 140-kD bound receptor.

Results

Initially, we attempted to identify a human fibroblast cell line that could be used for the isolation of type VI and other collagen receptors. We needed a cell line that would demonstrate rapid and promiscuous attachment to ECM components, including types I and III–VI collagen and fibronectin, and it was critical that the cells not synthesize a stable ECM or significant quantities of disulfide-bonded type VI collagen. As seen in Table I, both normal and transformed human mesenchymal cells attach to pepsinized collagen types I, III, IV, V, and VI, native nonpepsinized type VI collagen (results not shown), fibronectin, and laminin. All cells examined did synthesize soluble forms of native type VI collagen to varying degrees, but only the normal WI-38 cells assembled a stable detergent-insoluble ECM containing disulfide-bonded type VI collagen (Carter, 1982*b*, 1984). The transformed cells, however, maintained the ability to attach and spread on type VI collagen and other ECM components, indicating that they still possessed the full range of receptors. HT-1080 fibrosarcoma cells exhibited the most rapid and extensive adhesion to ECM components at room temperature (data not shown) as well as 37°C (Table I) and were selected for use in isolation of cell surface receptors for type VI collagen. Curiously, A204 cells appeared to exhibit a preference for type VI collagen when compared with other collagen types, even though type V collagen is the major collagenous protein synthesized by these cells (Alitalo et al., 1982).

Table I. Adhesion of Normal and Malignant Mesenchymal Cells to Protein-coated Surfaces

	Cell adhesion (percent of cells added)‡			
Adhesion surface*	A204	HT-1080	WI-38	WI-38 VA13
BSA	0.5	0.6	0.2	0.2
Concanavalin A§	38.5	89.9	85.5	52.3
Fibronectin	36.3	73.5	63.6	64.1
Laminin	5.6	36.1	65.9	53.7
Collagen				
I	6.8	53.4	68.4	57.6
III	9.0	33.0	64.1	57.9
IV	4.5	44.0	58.1	53.1
V	2.0	43.0	60.1	50.4
VI	29.4	52.8	65.8	62.7

* 48-well virgin styrene plates were coated with the indicated proteins (200 μl protein solution/well, 25 μg protein/ml for 2 h), then washed and blocked with 1% wt/vol heat-denatured BSA in PBS.

‡ Cells were labeled with ^{51}Cr, suspended by trypsin digestion, washed, placed in the protein-coated wells, and incubated for 30 min. The nonadherent cells were removed by washing and the bound cells were dissolved in SDS/NaOH and counted in a gamma counter. Cell adhesion = cpm (cells bound)/cpm (total cells added).

§ In most cases, cell attachment to concanavalin A represented the maximal number of cells that could bind to the adhesion surface.

Cell Adhesion to Collagen and Fibronectin Is Mediated by Independent Receptors

Previous results (Carter, 1982*a*) have shown that polyclonal antibodies that react specifically with fibronectin will inhibit cell adhesion to fibronectin but not type VI collagen. Thus, cell adhesion to type VI collagen does not proceed via interaction with fibronectin on the cell surface. The possibility that cell adhesion to type VI collagen and fibronectin might

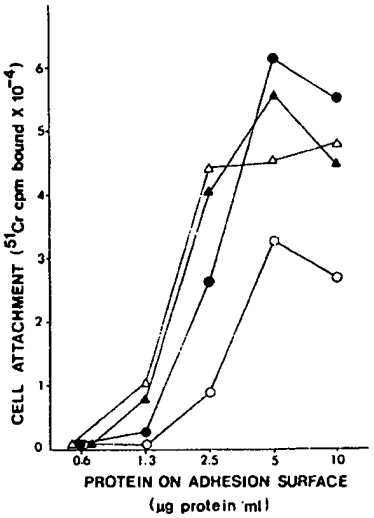


Figure 1. Inhibition of cell attachment to fibronectin and type VI collagen with soluble cell attachment domain of fibronectin. Fibronectin and type VI collagen were coated on plastic surfaces as described in Materials and Methods (0.6–10.0 μg protein/ml per well). The soluble cell attachment domain of fibronectin was isolated from thermolysin digests and added to the BSA-blocked adhesion surfaces as a competitive inhibitor of cell adhesion (final concentration of 1 mg/ml). HT-1080 cells were labeled with ^{51}Cr and added to the attachment assay, incubated for 30 min, and then washed to remove nonadherent cells. The adherent cells were dissolved in SDS/NaOH and counted in a gamma counter. (*Open triangles*) VI plus cell adhesion domain of fibronectin; (*solid triangles*) VI minus cell adhesion domain of fibronectin; (*open circles*) fibronectin plus cell adhesion domain of fibronectin; (*solid circles*) fibronectin minus cell adhesion domain of fibronectin.

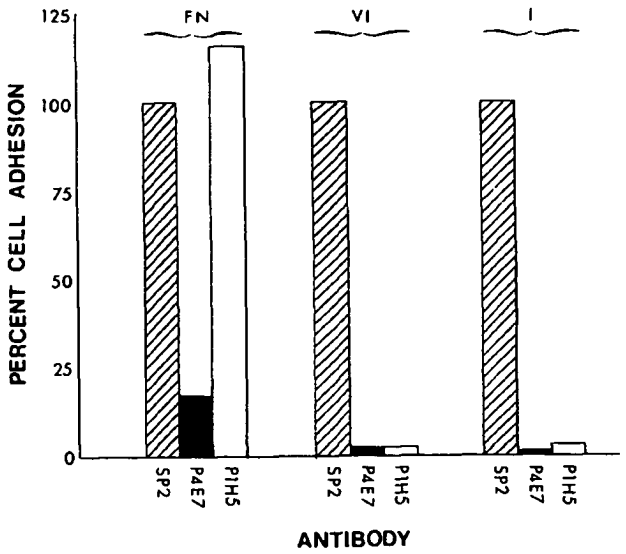


Figure 2. Inhibition of HT-1080 cell attachment to fibronectin, type I, and type VI collagens with class I (P4E7) and class II (P1H5) monoclonal antibodies. Cells were labeled with ^{51}Cr and incubated on plastic surfaces coated with fibronectin (FN), type VI collagen (VI), and type I collagen (I) in the presence of the indicated antibodies (100 μg antibody/ml final concentration) as described in Materials and Methods. After 30 min incubation the nonadherent cells were removed by washing and the adherent cells dissolved in SDS/NaOH and counted in a gamma counter. Total cells bound to each adhesion surface in the presence of SP2 cell culture supernatant alone, control, are indicated as 100%. The inhibitory effect of the class I (P4E7) and class II (P1H5) antibodies is calculated as a percentage of cell adhesion in the presence of SP2 cell culture supernatant alone.

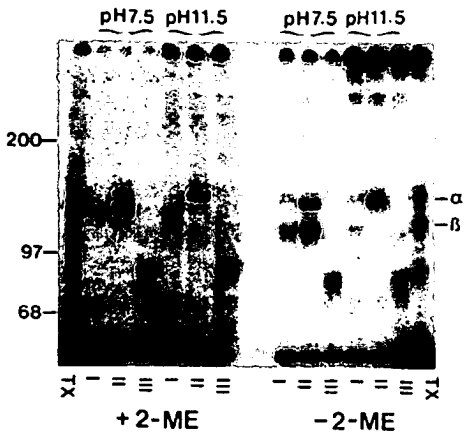


Figure 3. The effect of reducing agents and basic pH on the migration and association of the subunits of class I–III antigens. HT-1080 cells were surface labeled with radioactive iodine, then extracted with Triton X-100 detergent (*TX*). Aliquots of the soluble extract were immunoprecipitated with class I (P4E7), class II (P1H5), and class III (P1G12) monoclonal antibodies. Before immunoprecipitation, the extracts were either untreated or subject to temporary elevation of the pH to 11.5 followed by neutralization in order to dissociate the subunits. The immune precipitates were developed on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) and visualized by autoradiography. Migration of the α and β subunits of the class I and II antigens under nonreducing conditions are indicated at right.

involve distinct receptors was investigated by means of a competitive inhibition assay (Fig. 1). The data depicted in Fig. 1 show clearly that HT-1080 cell attachment to type VI collagen (or type I and III collagen, results not shown) was not inhibited by a proteolytic fragment of plasma fibronectin containing the cell attachment domain (140 kD, Fig. 1). In contrast, the cell attachment domain of plasma fibronectin (1 mg/ml) significantly inhibited HT-1080 cell attachment to fibronectin (54%). In addition, HT-1080 cell attachment to types I, III, and VI collagen were also unaffected by the arg-gly-asp-ser (RGDS)-containing peptides (data not shown). Similar inhibition results for cell adhesion to interstitial collagens with RGDS-containing peptides have been reported by Hayman et al. (1985). On the basis of these data it can be concluded that types I, III, and VI collagen and fibronectin do not compete for the same receptor binding site and that the collagen receptor may be RGDS independent.

Preparation of Monoclonal Antibodies that Specifically Inhibit the Interaction of Human Fibroblasts with Type VI Collagen

We attempted to identify the cell surface receptor(s) involved in attachment of mesenchymal cells to type VI collagen by preparing monoclonal antibodies to HT-1080 cells that would alter cell adhesion to ECM components. Monoclonal antibodies were successfully screened via a three-stage process. (*a*) Hybridomas producing antibodies that would react with whole HT-1080 cells in a solid-phase assay were identified. (*b*) Antibodies from HT-1080–positive hybridomas were screened for their ability to immunoprecipitate cell surface proteins from Triton X-100 detergent extracts of HT-1080 cells surface-labeled with radioactive iodine by the lactoperoxidase method. (*c*) Antibodies that would immunoprecipitate cell surface proteins were subsequently screened for their ability to inhibit or increase cell adhesion to type VI collagen. Since we were interested in obtaining antibodies to receptors specific for type VI collagen we also screened hybridoma supernatants for the ability to inhibit type I collagen, fibronectin, and laminin-mediated attachment as well. All positive hybridomas were subsequently cloned by limiting dilution to ensure monoclonality.

Using this screening process, we identified two classes of monoclonal antibodies that could alter HT-1080 cell attachment to type VI collagen. Class I monoclonal antibodies, represented here by P1B5, P2E6, and P4E7, all inhibited HT-1080 cell attachment to type VI and I collagens and, surprisingly, partially inhibited attachment to fibronectin (Fig. 2) and laminin as well (results not shown). Class I antibodies all precipitated the same cell surface protein. By SDS-PAGE, the class I antigens migrated as two bands with relative molecular masses of 147 and 125 kD under nonreducing conditions and were termed α and β, respectively (Fig. 3). In the presence of 2-ME, the α and β subunits reversed their relative order of migration but were poorly resolved and migrated with molecular masses of 130 and 135 kD, respectively. The fact that the α subunit of the class I antigen was found to migrate with lower relative molecular mass than the β subunit under reducing conditions was determined as fol-

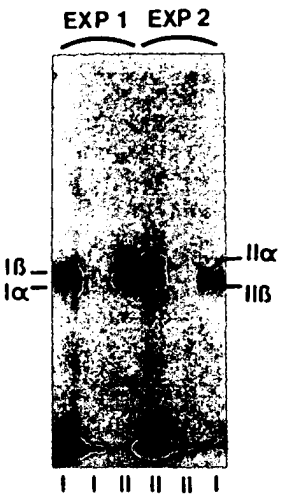


Figure 4. Sequential immunoprecipitation of class I and class II antigens. Experiment 1 (*EXP 1*): Triton X-100 detergent extracts were prepared from HT-1080 cells after surface labeling with radioactive iodine. An aliquot of the labeled extract was subject to three cycles of immunoprecipitation as follows. (*a*) Class I (P1B5) antibody was used to precipitate antigen from the extract. (*b*) The extract was then reprecipitated with class I antibody to ensure that the class I antigen was quantitatively removed. (*c*) The class I-free extract was then immunoprecipitated with class II (P1H5) antibody. The antigens from the three sequential immunoprecipitation steps were analyzed on SDS-PAGE gels (7%) in order (labeled I, I, and II, from left to right) in the presence of 2-ME followed by autoradiography. Migration of the α and β subunits of the class II (*right*) and class I (*left*) are indicated. Experiment 2 (*EXP 2*): the procedure was repeated as described above except that the sequence of precipitation steps was class II, class II, and class I (labeled II, II, and I from left to right).

lows. The α and β subunits were purified on a preparative SDS-PAGE gel under nonreducing conditions, where the subunits are resolved. The isolated subunits were again subjected to electrophoresis under reducing conditions (both side by side and remixed), where the α subunit migrated with a lower molecular mass than the β subunit (results not shown).

Class II monoclonal antibodies, represented by clone P1H5, inhibited cell adhesion to type VI and I collagens (and type III, IV, and V collagens, data not shown) only and not to fibronectin (Fig. 2) or laminin (data not shown). The class II antibodies precipitated a single cell surface protein with two subunits with molecular masses of 145 and 125 kD for the α and β subunits, respectively, under nonreducing conditions. In the presence of 2-ME, the α and β subunits migrated with molecular masses of 140 and 135 kD, respectively. The data obtained from inhibition of cell attachment studies with class II antibodies strongly suggested that attachment to collagen was mediated by this class II receptor.

A third class of monoclonal antibody, termed class III, and represented by clones P1G12 and P3H9, either increased cell adhesion to collagen (under conditions of minimal cell attachment) or had no effect (similar to the SP2 control in Fig. 2, when attachment approached maximum), and immunoprecipitated an antigen that migrated as a diffuse single band on SDS-PAGE gels with a relative molecular mass of 90 kD under reducing conditions and 80 kD under nonreducing conditions (Fig. 3). The class III antigen appeared to be similar to an 80–90-kD cell surface glycoprotein we previously reported as binding to type VI collagen (Carter and Wayner-Carter, 1986). Therefore, antibodies reactive with class III antigen were selected for further study.

As previously indicated (Fig. 3), class I–III antigens could be labeled by lactoperoxidase-catalyzed iodination, indicating that they are present on the cell surface. These proteins could also be metabolically labeled with radioactive amino acids (Fig. 6) and glucosamine (data not shown), indicating that they are cell-synthesized glycoproteins. The membrane localization of all three classes of antigen was further established by immunofluorescence microscopy (data not shown).

These results indicated that HT-1080 cell adhesion to fibronectin and collagen could be differentiated on the basis of inhibition with the class I and class II monoclonal antibodies. They further suggested that cell adhesion to collagen was mediated by both class I and II antigens and that adhesion to fibronectin was mediated, at least in part, by the class I antigen. Results to be presented below and elsewhere (Wayner, E. A., and W. G. Carter, work in progress) suggested that there are at least two distinct fibronectin receptors. The relationship of the class I antigen to the fibronectin receptor described by others (Pytela et al., 1985) will be discussed in detail (see below).

Comparison of Class I and II Antigens

Sequential immunoprecipitation of the class I and II antigens with the appropriate antibodies indicated that neither antibody class recognized antigen from the other class (Fig. 4). Similar comparisons were also made between class III and class I or II antigens using sequential immunoprecipitation, with no indication of cross-reactivity between the antibody classes. Immunoprecipitation of the class II antigen after temporary adjustment of the pH from 7.5 to 11.5 and back again in order to dissociate the subunits resulted in precipitation of only the α subunit (Fig. 3). Thus, the class IIβ subunit is noncovalently associated with the α subunit and is not recognized by the class II antibody. Subunit-dissociating conditions (pH 11.5, see Fig. 3, SDS or SDS plus 2-ME) either failed to dissociate the α and β subunits of the class I antigens or denatured the epitope recognized by the antibody. Thus, the α and β subunits of the class I antigens appeared to be more tightly associated than the subunits of the class II antigen or the class I antibody (P1B5) reacted with both subunits. Regardless, this method did not enable us to conclusively identify which subunit contained the epitope recognized by the class I antibody. However, since anti-class IIα did not cross-react with class I (Fig. 4) and since the class Iβ and IIβ subunits appeared to be identical (see below) it can be inferred that P1B5 anti-class I probably reacts with either an α-specific or an α-β complex. This possibility was confirmed by the pulse-chase labeling described below.

The β subunits of the class I and II antigens co-migrated under both reducing and nonreducing conditions (Fig. 3) in contrast to the α subunits, suggesting that the β subunits may be structurally related proteins. This possibility was confirmed by proteolytic digestion of the α and β subunits from both classes of antigen using V8 protease followed by comparison of the peptides by the method of Cleveland et al. (1977). As seen in Fig. 5, the peptide maps of the β subunits were virtually identical, suggesting a high degree of amino acid sequence homology. In contrast, the peptide maps of the α subunits were clearly distinguishable. Curiously, the class IIα subunit did exhibit some degree of homology to both β subunits. The structural similarities of the class I and II antigens suggested that one antigen may be a metabolic precursor of the other. This possibility was further analyzed by following the kinetics of synthesis of the two antigens by pulse-chase labeling studies with radioactive methionine

Figure 5. Comparison of the peptide maps of the α and β subunits of class I and II antigens after digestion with V8 protease. The α and β subunits of class I and II antigens were isolated from Triton X-100 extracts of surface-labeled HT-1080 cells by immunoprecipitation and preparative SDS-PAGE under nonreducing conditions. Each isolated subunit was digested with *S. aureus* V8 protease, followed by comparison of the peptide maps on SDS-PAGE gels (20% acrylamide), and autoradiography.

(Fig. 6). A number of specific points were made clear by these studies. (*a*) Throughout the duration of the study (0–30 h), both the α and β subunits of class I and II antigens exhibited similar kinetics for methionine incorporation, as observed by immunoprecipitation. Thus, there was no indication that either class I or II antigen was a precursor to the other. (*b*) A metabolic precursor to the class I antigen, labeled preI in Fig. 6, was observed during the first 2 h of the chase period. The preI form did not co-migrate with any of the common β subunits in either class I or II and was not precipitated by the α-specific class II antibody. Therefore, preI probably corresponded to the precursor to the Iα subunit before association with the common β subunit. Thus, the PIB5 class I monoclonal antibody must react with the Iα subunit, not the Iβ or α–β combination. (*c*) We did not observe that the class IIα subunit was a precursor to the class IIβ subunit. Even after a prolonged chase period (30 h), there was no observed decrease of label in IIα corresponding to an increase in IIβ. The epitope for the class II antibody was localized to the α subunit (Fig. 3). Thus, the observed 30–60-min delay in precipitation of the IIβ subunit probably reflects the posttranslational association of the IIβ subunit with the IIα subunit. This delay in posttranslational association was also observed for the association of the Iα and Iβ subunits.

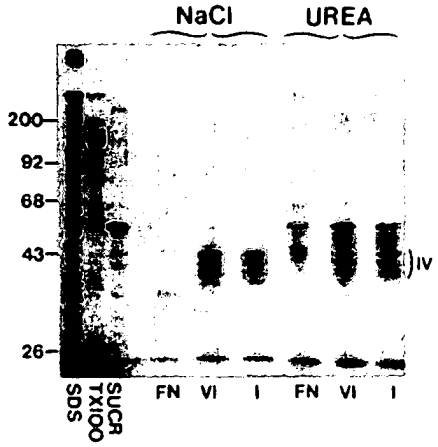


Figure 7. Identification of the peripheral cell surface receptors for type I and type VI collagen by affinity chromatography of HT-1080 cell extracts. Surface-labeled cells were differentially extracted as described in Materials and Methods. The three extracts, peripheral (*SUCR*), membrane (*TX*), and SDS-soluble (*SDS*) were compared. The extract containing the labeled peripheral components that did not require detergent for solubilization was divided into equal aliquots and chromatographed on type I collagen-Sepharose (*I*), type VI collagen-Sepharose (*VI*), and fibronectin-Sepharose (*FN*). Unbound protein was removed by washing and bound protein eluted first with 200 mM NaCl (*NaCl*) and second with 6 M urea (*UREA*). The eluted material was analyzed on SDS-PAGE gels (12% acrylamide) by autoradiography. Migration of standard proteins is indicated at left.

Identification of Multiple Cell Surface Receptors for Type VI Collagen and Fibronectin by Affinity Chromatography

Surface-labeled HT-1080 cells were sequentially extracted with buffer containing: (*a*) isotonic sucrose, with no detergent, to solubilize peripheral components that were not intrinsic to cellular membranes; (*b*) Triton X-100 detergent, to solubilize membrane components; and (*c*) SDS to dissolve the residue (see Fig. 7). Greater than 70% of the total cell protein was soluble in the absence of detergent, including labeled proteins in the molecular mass range of 30–60 kD (Fig. 7). Extraction with Triton X-100 detergent released multiple

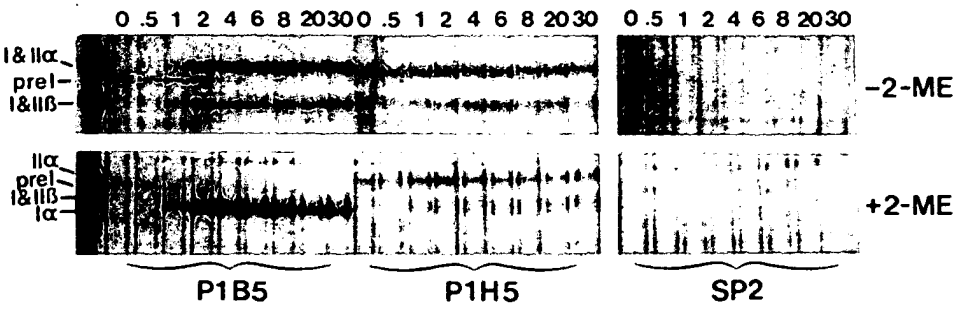


Figure 6. Analysis of the kinetics of synthesis of class I and II antigens by pulse-chase metabolic labeling. HT-1080 cells were pulse labeled with radioactive methionine for 1 h and then chased with nonradioactive methionine for the indicated time periods (0, 0.5 h, etc.). Time point 0 corresponds to the end of the pulse-labeling period. Triton X-100 detergent-soluble extracts were prepared from the cells and immunoprecipitated with class I (*PIB5*) and class II (*PIH5*) monoclonal antibodies or control culture supernatant (*SP2*) and then analyzed on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) followed by fluorography. Only relevant regions of the fluorographs are shown. The 30-h SP2 control (*+ 2-ME*) time point is not shown. Migration of the α and β subunits of the class I and II subunits are indicated at left. Migration of precursor forms of the class I antigens are labeled *preI*. Migration of ^{125}I surface-labeled class I and II antigens from HT-1080 cells are shown in the gel lanes at far left.

labeled proteins that required detergent for solubilization and co-migrated with the antigens precipitated with the class I and III monoclonal antibodies (Fig. 7). The two extracts containing the peripheral and membrane components solubilized with Triton X-100 detergent were each diluted in buffer containing BSA (0.5% wt/vol) and Triton X-100 detergent and then passed over BSA-Sepharose to remove proteins that might bind nonspecifically to subsequent affinity columns. Gelatin-Sepharose, or denatured collagen, was also included as a further control to identify nonspecifically bound proteins and gave results similar to BSA-Sepharose (results not shown). Aliquots of the extracts were chromatographed on fibronectin-, type I collagen-, and type VI collagen-Sepharose. Each column was washed with buffer containing Triton X-100 to remove unbound components. Bound proteins were eluted with wash buffer containing, first, 200 mM NaCl, and second, 6 M urea. A two-stage elution protocol was used because we assumed that the various components, particularly the hydrophobic membrane proteins, might exhibit weak interactions with the affinity columns after solubilization with detergents and would be denatured by an ionic environment. Physiological salt concentrations have previously been reported to disrupt receptor-collagen interactions (Mollenhauer and von der Mark, 1983).

Three proteins with molecular masses of 38, 42, and 45 kD from the peripheral cell extracts (Fig. 7) bound to both type I and VI collagen but not fibronectin and were termed class IV receptors. These proteins could be partially eluted with NaCl. A protein of 56 kD bound to all the affinity columns, exhibiting no binding specificity, and required urea for elution. These results suggest that the class IV receptors bind preferentially to both type I and VI collagens and that these proteins are peripherally associated cell surface components and not intercalated into the plasma membrane.

The Triton X-100 detergent extract of HT-1080 cells contained two labeled cell surface proteins or groups of proteins that bound reproducibly to both type I and type VI collagen with molecular masses of approximately 140 kD (termed class I receptor) and 80–90 kD (termed class III receptor) when subjected to electrophoresis under reducing conditions (Fig. 8). In contrast, a single labeled protein of 140 kD bound to fibronectin. These results indicated that the class III and IV receptors bond only to collagen, while class I–like receptors, possibly identical, bond to both collagen and fibronectin. The failure of any of these receptors to bind to gelatin indicated that fibronectin, possibly present in the cell extract, did not link the receptors to the native collagen-Sepharose. It also indicated that the receptors preferentially bound to native collagen conformations.

Identification of the Collagen and Fibronectin Receptors as Class I and III Antigens

The possibility that any of the class I–III antigens defined by the monoclonal antibodies may be identical to the cell surface proteins bound to the affinity columns was examined by (*a*) comparing the mobility of the various components on SDS-PAGE gels under both reducing and nonreducing conditions (Fig. 9) and (*b*) immunoprecipitation of the affinity-purified receptors with the monoclonal antibodies (Fig. 10). The class I antigen co-migrated with the class I receptor that bound to both fibronectin and collagens. The class I receptor exhibited the characteristic alterations in migration of the α and β subunits of the class I antigen under reducing and nonreducing conditions in contrast to the distinct migration of the class II antigen as previously described (Fig. 3). In addition, the class I antibody, but not the class II antibody, immunoprecipitated the class I receptors bound to both fibronectin

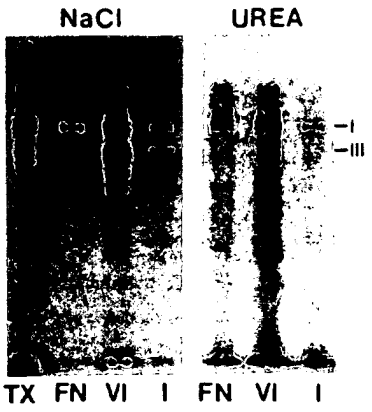


Figure 8. Identification of membrane receptors for type I and type VI collagen and fibronectin by affinity chromatography of HT-1080 cell extracts. Surface-labeled cells were differentially extracted as described in Fig. 7 and Materials and Methods. The labeled membrane components that required Triton X-100 detergent for solubilization (*TX*) were extracted, chromatographed, and analyzed as described in Fig. 7. The quantity of radioactive material eluted with urea was minimal compared with material eluted with NaCl, and was therefore given five times the exposure period of the NaCl-eluted material.

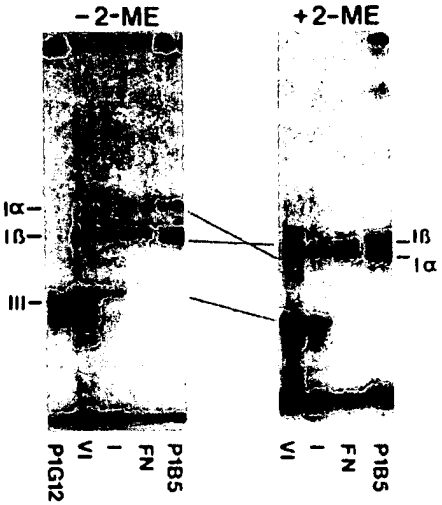


Figure 9. Comparison of the class I and III antigens to the affinity-purified receptors for fibronectin, type I, and type VI collagen. Surface-labeled HT-1080 cells were differentially extracted as described in Fig. 7 and Materials and Methods. The Triton X-100 detergent extract was used for affinity purification of receptors for fibronectin (*FN*), type I collagen (*I*), and type VI collagen (*VI*) as described in Fig. 8, using salt elution. The detergent extract was also used for immunoprecipitation with class I (P1B5) and class III (P1G12) antibodies. The relative migration of the isolated receptors and antigens were compared on SDS-PAGE gels (7% acrylamide) in the presence and absence of 2-ME (*+ or − 2-ME*) after autoradiography.

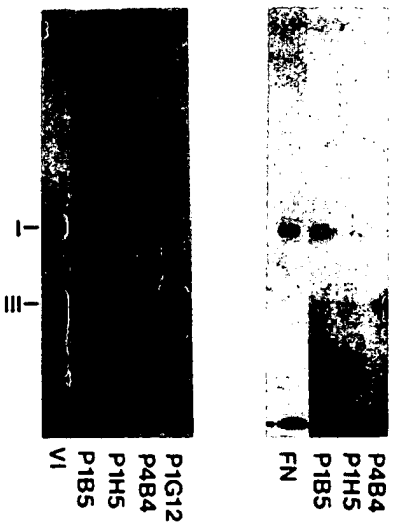


Figure 10. Immunoprecipitation of affinity-purified receptors for fibronectin, type I, and type VI collagen with class I and III monoclonal antibodies. Receptors for type VI collagen (*VI*) and fibronectin (*FN*) were affinity purified from Triton X-100 extracts of surface-labeled HT-1080 cells as described in Fig. 5. The receptors were then immunoprecipitated with the following antibodies: class I, P1B5; class II, P1H5 and P4B4; and class III, P1G12. The immunoprecipitates and the receptors were compared on SDS-PAGE gels (7% acrylamide) in the absence of 2-ME by autoradiography.

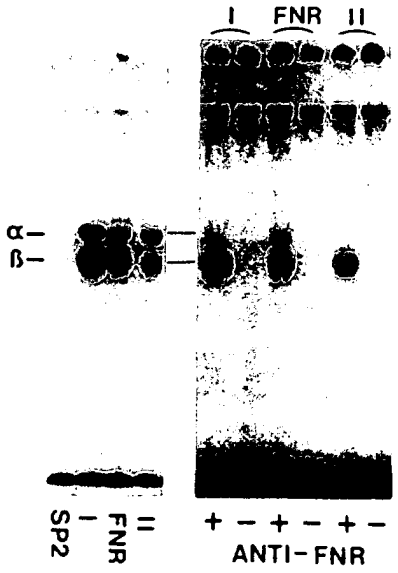


Figure 11. Relation of the class I and II receptors to the FNR. (*Left*) Triton X-100 detergent-soluble extracts were prepared from surface-labeled HT-1080 cells and immunoprecipitated with the class I, II, and anti-FNR antibodies or control SP2 culture supernatant. The antigens were fractionated on SDS-PAGE gels (8%) in the absence of 2-ME in order to resolve the α and β subunits. The migration of the subunits are indicated at left. (*Right*) The class I, II, and fibronectin receptors were purified by immunoprecipitation as above, and then the α and β subunits and antigen–antibody complexes were dissociated in SDS at 100°C. The dissociated subunits from each receptor were then reprecipitated with anti-FNR and examined on SDS-PAGE gels in the absence of 2-ME. The anti-FNR immunoprecipitated the β subunit from all three receptors.

and collagen (Fig. 10). These results indicated that the class I receptor that bound to fibronectin and collagen contained detectable levels of the class I antigen. The fact that the class I receptor could be immunoprecipitated from proteins affinity purified on type VI collagen and fibronectin confirmed the cell attachment data (Fig. 2) and strongly suggested the existence of fibroblast cell surface receptors capable of interacting with multiple ECM components. We are currently investigating whether the class I receptor can interact with RGD-containing peptides. In the presence of Triton X-100 or zwitterionic detergents such as Empigen BB, the class II receptor did not appear to bind native collagen (See Discussion).

As seen in Fig. 9, the class III antigen was found to co-migrate with the class III collagen receptor and this receptor was immunoprecipitated with the class III antibody (Fig. 10). A protein band migrating at ~90 kD was not immunoprecipitated with the class III antibody and may represent an additional receptor unrelated to the class III antigen. This possibility is currently under further investigation. We also found that the isolated class III receptor degraded on storage, with generation of lower molecular mass degradation products, primarily in the 60-kD range. These degradation products could not be immunoprecipitated with the class III antibodies.

The Relation of Class I Antigen to the Previously Described Fibronectin Receptor

Both the class I and II receptors bore remarkable structural similarities to the fibronectin receptor (FNR) isolated from osteosarcoma cells by Pytela et al. (1985). However, both the class I and II receptors exhibit different binding specificities, based on inhibition of cell adhesion, then ascribed to the FNR. To facilitate a comparison of the class I and II receptors to the FNR, we obtained rabbit polyclonal antibodies prepared against the FNR, termed anti-FNR. The relationships of the class I and II antigens to the FNR were examined in the following series of experiments. (*a*) Immunoprecipitation of Triton X-100 detergent extracts prepared from surface-labeled HT-1080 cells was performed with class I and II monoclonal antibodies and anti-FNR. On SDS-PAGE gels, under reducing (Fig. 11) and nonreducing conditions (results not shown), the migration of the α and β subunits of the class I and FNR antigens were virtually identical (Fig. 11). As expected, under nonreducing conditions, only the β subunit of the class II antigens co-migrated with the other β subunits (data not shown). Thus, on the basis of subunit migration, the class IIα appeared to be distinct from the FNRα. In contrast, the class Iα subunit was similar to the α subunit of the FNR. (*b*) Sequential immunoprecipitation experiments revealed that the β subunits of all three antigens (I, II, and FNR) were identical. The class I and II antigens were purified by immunoprecipitation, denatured to separate the α and β subunits, and then reprecipitated with anti-FNR. As seen in Fig. 11, anti-FNR immunoprecipitated the β subunits of the class I and II antigens, suggesting that all three antigens bear the same β subunit in support of our peptide-

mapping studies (Fig. 5). Further, we compared the β subunits of the class I and II receptors and FNR by peptide analysis using the method of Cleveland et al. (1977) and detected extensive homology in all three β subunits (results not shown). Anti-FNR did not immunoprecipitate the Iα subunit, suggesting that the class Iα and FNRα antigens were not identical (Fig. 11). However, in control experiments, anti-FNR reacted only weakly with its own denatured FNRα subunit (Fig. 11), suggesting that anti-FNR was primarily β subunit specific. The possibility that anti-FNR might react more specifically with nondenatured FNRα or class Iα was evaluated by preclearing experiments. However, without prior dissociation of the α and β subunits, anti-FNR was found to partially immunoprecipitate both class I and II antigens as well as additional proteins containing similar α–β subunit structures, as would be expected due to the common β subunit specificity (results not shown). Because the anti-FNR antibodies reacted primarily with the common β subunit, further comparison of the class Iα and IIα subunits with the FNRα were not feasible using this antibody. (*c*) To compare the α subunits of the class I and II receptors to FNRα, we purified FNR from octylglucoside extracts of surface-labeled HT-1080 cells by chromatography of the extracts on insoluble fibronectin. The bound FNR was not eluted with a nonadhesive peptide, thr-lys-pro-arg (Tuftsin), but was specifically eluted with the adhesion-active peptide, gly-arg-gly-asp-ser, as described by Pytella et al. (1985). This affinity-purified FNR co-migrated with the class I receptor on SDS-PAGE gels under both reducing and nonreducing conditions. However, the RGDS peptide–eluted FNR did not react with the class I monoclonal antibody but did react with the β subunit–specific anti-FNR antibodies (results not shown).

In summary, based on the inhibition of cell adhesion data (Fig. 2) and the results with the β subunit–specific anti-FNR (Fig. 11) and affinity-purified FNR prepared as described by Pytela et al. (1985), we conclude that: (*a*) the class I, II, and FNR antigens are all members of a related family of cell surface ECM receptors that possess common β subunits; and (*b*) the α subunits of the class I, II, and FNR antigens are immunologically and functionally unique. Thus, class I and II represent two new members of the ECM receptor family that possess α subunits which are functionally distinct from the previously described FNR.

Discussion

Many normal human mesenchymal cells synthesize a complex ECM which contains multiple detergent-insoluble adhesive glycoproteins, including fibronectin, type VI collagen, and GP250 (Carter, 1982*a*, *b*). We have previously proposed that these proteins may function cooperatively to mediate cell adhesion (Carter, 1982*a*). To further clarify the mechanisms of adhesion to and assembly of the ECM, we have identified four classes of cell surface protein receptors, classes I–IV, that interact with collagen and/or fibronectin as determined by: (*a*) inhibition of cell adhesion with monoclonal antibodies to receptors and/or (*b*) affinity chromatography on immobilized ECM proteins.

Class II Antigen: A Collagen-specific Receptor Involved in Cell Adhesion

Although we initiated these studies to identify the receptor for type VI collagen, we did not obtain any antibodies that specifically inhibited cell attachment to type VI. All the class II antibodies that inhibited attachment to type VI collagen also inhibited attachment to other collagens, but not to fibronectin, laminin, or lectin-coated surfaces. However, very little class II antigen bound to native collagen columns under the present affinity chromatography conditions. In contrast, the promiscuous class I receptor exhibited a much more stable interaction with ligands and was shown to bind to both collagen and fibronectin in support of the monoclonal antibody inhibition data. At the present time we have no explanation for the difference in the stability of class I and II receptor–ligand interaction. One possible explanation might simply be that the association of the class II α and β subunits is unstable. For example, high pH dissociated the class II α and β subunits, but not the class I α and β subunits. We also observed that the α subunit–specific epitope recognized by the class II antibodies was denatured by Empigen BB, a zwitterionic detergent. This detergent had no effect on the epitopes recognized by the class I antibodies. Taken together, these results suggest that the class II receptor is unstable and may not retain its collagen-binding conformation after solubilization with most detergents. Alternatively, the naturally occuring receptor for the class II antigen may be a complex of collagen with some other component of the ECM, such as fibronectin or glycosaminoglycan. Both possibilities are currently being investigated. The class II antigen appears to be a new member of the family of adhesion receptors with common β subunits and unique α subunits, which confer binding specificity for multiple components of the ECM involved in cell adhesion.

Class I Receptor: A Promiscuous Cell Adhesion Receptor for Multiple Components of the ECM

The class I receptor bound to fibronectin in affinity chromatography experiments and reacted with the class I monoclonal antibodies which inhibited cell adhesion to fibronectin. These results indicate that HT-1080 cell adhesion to fibronectin is mediated, at least in part, by the class I receptor. However, the class I receptor also bound to type I and VI collagens and cell adhesion to collagen and laminin were also inhibited by class I monoclonal antibodies. Because of their obvious biochemical similarities, it was necessary to investigate the relationship of the class I and II antigens to the FNR described by Pytela et al. (1985). Polyclonal antibodies specific to the FNR were found to cross-react with the β subunits of both the class I and II receptors, indicating that they are all member of the same ECM receptor family. However, no cross-reaction of the anti-FNR antibodies were observed with the α subunits of either the class I or II receptors after the α subunits were dissociated from the common β subunits. In addition, neither the class I nor II antibodies reacted with the FNR isolated by RGDS peptide elution from fibronectin-Sepharose. Thus, both the class I and II receptors are new members of the ECM receptor family and shall be referred to as ECMRI and II in the future. In support of the observed differences between the class I and II receptors and FNR, we have recently isolated a new class of monoclonal antibody, referred to as class VI. The class VI monoclonal antibodies specifically inhibit cell adhesion to fibronectin and immunoprecipitate an antigen containing the common β subunit and a third unique α subunit (Wayner, E. A., and W. G. Carter, work in progress). The relationship of the class VI receptor to the FNR is being investigated. However,

it seems clear from the present findings that attachment of cells to fibronectin may involve multiple independent receptors (class I, VI, and/or the FNR). Heterogeneity in the binding specificity of the FNR has already been suggested by Horwitz et al. (1985), who reported that the CSAT antibody inhibited cell adhesion to both laminin and fibronectin. It should be interesting to determine the amino acid sequences present in collagen and fibronectin recognized by the class I receptor. It is possible that this receptor either recognizes a sequence common to collagen and fibronectin, or is capable of interacting with multiple distinct adhesion-promoting sequences. Conceivably, such promiscuity in the binding specificity of the class I receptor from human fibrosarcoma HT-1080 cells may not be exhibited by this receptor in all cells. Further, differential expression of the three receptors, class I, II, and VI, by normal versus transformed mesenchymal cells, epithelial cells, and platelets have been observed (Wayner, E. A. and W. G. Carter, manuscript in preparation). Altered specificity and/or inappropriate expression of specific receptors for ECM components are attractive possible explanations for variation in the metastatic potential of malignant cells or tissue-homing specificity.

Recently, various groups have identified single or even families of related proteins that probably mediate cell adhesion to fibronectin, vitronectin, and laminin in human, rodent, and chicken cells. It seems clear that the collagen receptor, class II, and the promiscuous class I ECM receptor are new members of the family of ECM receptor proteins. Although we were not successful in isolating a specific receptor involved in adhesion to type VI collagen, we are still assuming that interaction of mesenchymal cells with different collagen types occurs at the level of a specific membrane receptor. Conceivably, interaction of a cell with a particular collagen type may depend on additional interactions with globular domains residing outside of the pepsin-resistant, collagenous domains.

Class III and IV Receptors

In contrast to the inhibitory effect of class I and II antibodies on cell adhesion, class III antibodies stimulated attachment or had no effect. The class III antibodies immunoprecipitated a cell surface glycoprotein of 80–90 kD that could be metabolically or cell surface–labeled and was distinct from either class I or II antigens. We have previously described a similar cell surface glycoprotein which is resistant to extraction with detergent, suggesting a possible interaction with the ECM or cytoskeleton (Carter, 1982*a*), and which specifically binds to type VI collagen in affinity chromatography experiments (Carter and Wayner-Carter, 1986). We have also recently obtained evidence that class III shows a preferential association with cytoskeletal components (Carter, 1985; Carter, W. G., and E. A. Wayner, manuscript in preparation). These results suggest that the class III antigen may associate with type VI collagen in the ECM and perhaps with the cytoskeleton on the cytoplasmic side of the membrane. We have also purified three cell surface proteins, termed the class IV receptors, with subunit molecular masses of 38, 42, and 45 kD which bind to both type I and VI collagen. The class IV receptors differ from I–III in that they do not require detergent for solubilization from the membranes and are therefore probably peripherally associated with the cell surface. Kurkinen et al. (1984) have described similar proteins, and a major 47-kD protein, termed "colligin," which binds gelatin as well as native type IV collagen. The relation of the class IV receptors to these and other related proteins will be described elsewhere (Carter, W. G., and E. A. Wayner, manuscript in preparation).

This work was supported by grant BC-419 from the American Cancer Society and by grant ROI-CA38801 from the National Institutes of Health.

Received for publication 30 December 1986, and in revised form 6 May 1987.

References

Akiyama, S. K., S. S. Yamada, and K. M. Yamada. 1986. Characterization of a 140-kD avian cell surface antigen as a fibronectin-binding molecule. *J. Cell Biol.* 102:442–448.

Alitalo, K., R. Myllyla, H. Sage, P. Pritzl, A. Vaheri, and P. Bornstein. 1982. Biosynthesis of type V procollagen by A204, a human rhabdomyosarcoma cell line. *J. Biol. Chem.* 257:9016–9024.

Brown, P. J., and R. L. Juliano. 1985. Selective inhibition of fibronectin-mediated cell adhesion by monoclonal antibodies to a cell-surface glycoprotein. *Science (Wash. DC).* 228:1448–1451.

Brown, P. J., and R. L. Juliano. 1986. Expression and function of a putative cell surface receptor for fibronectin in hamster and human cell lines. *J. Cell Biol.* 103:1595–1603.

Carter, W. G. 1982*a*. The cooperative role of the transformation sensitive glycoproteins, GP140 and fibronectin, in cell attachment and spreading. *J. Biol. Chem.* 257:3249–3257.

Carter, W. G. 1982*b*. Transformation-dependent alterations in glycoproteins of the extracellular matrix of human fibroblasts: characterization of GP250 and the collagen-like GP140. *J. Biol. Chem.* 257:13805–13815.

Carter, W. G. 1984. The role of intermolecular disulfide bonding in deposition of GP140 in the extracellular matrix. *J. Cell Biol.* 99:105–114.

Carter, W. G. 1985. Characterization of GP80, a cell surface receptor for GP140 of the extracellular matrix. *Fed. Proc.* 44:477. (Abstr.)

Carter, W. G., and S. Hakomori. 1981. A new cell surface, detergent-insoluble glycoprotein matrix of human and hamster fibroblasts: the role of disulfide bonds in stabilization of the matrix. *J. Biol. Chem.* 256:6953–6960.

Carter, W. G., and E. A. Wayner-Carter. 1986. Characterization of plasma membrane receptors for type VI collagen of the extracellular matrix. *J. Cell Biol.* 103(5, pt. 2):259a. (Abstr.)

Chapman, A. E. 1984. Characterization of a 140Kd cell surface glycoprotein involved in myoblast adhesion. *J. Cell. Biochem.* 259:109–121.

Chiang, T. M., and A. H. Kang. 1982. Isolation and purification of collagen ã1(I) receptor from human platelet membrane. *J. Biol. Chem.* 257:7581–7586.

Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.

Dambsky, C. H., K. A. Knudsen, D. Bradley, C. A. Buck, and A. F. Horwitz. 1985. Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. *J. Cell Biol.* 100:1528–1539.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble forms of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.

Gardner, J. M., and R. O. Hynes. 1985. Interaction of fibronectin with its receptor on platelets. *Cell.* 42:439–448.

Hayman, E. G., M. D. Pierschbacher, and E. Ruoslahti. 1985. Detachment of cells from culture substrate by soluble fibronectin peptides. *J. Cell Biol.* 100:1948–1954.

Heller-Harrison, R. A., and W. G. Carter. 1984. Pepsin-generated type VI collagen is a degradation product of GP140. *J. Biol. Chem.* 259:6858–6864.

Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134–2144.

Hynes, R. O. 1973. Alteration of cell-surface proteins by viral transformation and by proteolysis. *Proc. Natl. Acad. Sci. USA.* 70:3170–3174.

Jander, R., J. Rauterberg, B. Voss, and D. B. Bassewitz. 1981. A cysteine-rich collagenous protein from bovine placenta: isolation of its constituent polypeptide chains and some properties of the non-denatured protein. *Eur. J. Biochem.* 114:17–25.

Kotite, N. J., and L. W. Cunningham. 1986. Specific adsorption of a platelet membrane glycoprotein by human insoluble collagen. *J. Biol. Chem.* 261: 8342–8347.

Kurkinen, M., A. Taylor, J. I. Garrels, and B. L. M. Hogan. 1984. Cell surface-associated proteins which bind native type IV collagen or gelatin. *J. Biol. Chem.* 259:5915–5922.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Miller, E., and R. Rhodes. 1982. Preparation and characterization of the different types of collagen. *Methods Enzymol.* 82:33–64.

Mollenhauer, J., and K. von der Mark. 1983. Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:45–50.

Mollenhauer, J., J. A. Bee, M. A. Lizarbe, and K. von der Mark. 1984. Role

of anchorin CII, a 31,000-mol-wt membrane protein, in the interaction of chondrocytes with type II collagen. *J. Cell Biol.* 98:1572–1579.
Nagata, K., M. J. Humphries, K. Olden, and K. M. Yamada. 1985. Collagen can modulate cell interactions with fibronectin. *J. Cell Biol.* 101:386–394.
Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman and Co., San Francisco. 351–373.
Parikh, I., S. March, and P. Cuatrecasas. 1974. Topics in the Methods of Substitution Reactions with Agarose. Vol. 34. 77–101.
Pierschbacher, M. D., and E. Ruoslahti. 1984*a*. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
Pierschbacher, M. D., and E. Ruoslahti. 1984*b*. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985–5988.
Plow, E. F., J. C. Loftus, E. G. Levin, D. S. Fair, D. Dixon, J. Forsyth, and M. H. Ginsburg. 1986. Immunologic relationship between platelet membrane glycoprotein GPIIb/IIIa and cell surface molecules expressed by a variety of cells. *Proc. Natl. Acad. Sci. USA.* 83:6002–6006.
Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoproteins IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science (Wash. DC).* 28:1559–1562.
Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.
Rauvala, H., W. G. Carter, and S. I. Hakomori. 1981. Studies on cell adhesion and recognition. 1. Extent and specificity of cell adhesion triggered by carbohydrate-reactive proteins (glycosidases and lectins) and by fibronectin. *J. Cell Biol.* 88:127–137.
Rubin, K., M. Hook, B. Obrink, and R. Timpl. 1981. Substrate adhesion to rat hepatocytes: mechanism of attachment of collagen substrates. *Cell.* 24:463–470.
Saito, Y., T. Imada, J. Takagi, T. Kikuchi, and Y. Inada. 1986. Platelet factor XIII, the collagen receptor? *J. Biol. Chem.* 261:1355–1358.
Santoro, S. A. 1986. Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. *Cell.* 46:913–920.
Sekiguchi, K., and S. I. Hakomori. 1983. Domain structure of human plasma fibronectin. *J. Biol. Chem.* 258:3967–3973.
Taggart, R. T., and I. M. Samloff. 1983. Stable antibody-producing murine hybridomas. *Science (Wash. DC).* 219:1228–1230.
Timpl, R., H. Rohde, P. G. Robey, S. I. Rennard, J. M. Foidart, and G. R. Martin. 1979. Laminin- a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.
Wylie, D. E., C. H. Dambsky, and C. A. Buck. 1979. Studies on the function of cell surface glycoproteins. *J. Cell Biol.* 80:385–402.
Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leingruber, and M. Weigle. 1972. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science (Wash. DC).* 178:871–872.

The EMBO Journal vol.9 no.3 pp.765-770, 1990

Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain

F.Hogervorst, I.Kuikman, A.E.G.Kr.von dem Borne and A.Sonnenberg

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

Communicated by H.L.Ploegh

The $\alpha 6\beta 4$ complex is a member of the integrin superfamily of adhesion receptors. A human keratinocyte lambda gt11 cDNA library was screened using a monoclonal antibody directed against the $\beta 4$ subunit. Two cDNAs were selected and subsequently used to isolate a complete set of overlapping cDNA clones. The $\beta 4$ subunit consists of 1778 amino acids with a 683 amino acid extracellular domain, a 23 amino acid transmembrane domain and an exceptionally long cytoplasmic domain of 1072 residues. The deduced amino-terminal sequence is in good agreement with the published amino-terminal sequence of purified $\beta 4$. The extracellular domain contains five potential N-linked glycosylation sites and four cysteine-rich homologous repeat sequences. The extracellular part of the $\beta 4$ subunit sequence shows 35% identity with other integrin β subunits, but is the most different among this class of molecules. The transmembrane region is poorly conserved, whereas the cytoplasmic domain shows no substantial identity in any region to the cytoplasmic tails of the known β sequences or to other protein sequences. The exceptionally long cytoplasmic domain suggests distinct interactions of the $\beta 4$ subunit with cytoplasmic proteins.

Key words: cDNA sequence/integrin/$\beta 4$ subunit

Introduction

Integrins are $\alpha\beta$ subunit cell surface heterodimers which mediate cell-cell and cell-matrix interactions (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The integrin family is divided into three subfamilies; the VLA protein family (Hemler *et al.*, 1987), the Leu-Cam proteins (Springer *et al.*, 1987) and the cytoadhesins (Ginsberg *et al.*, 1988). These subfamilies are characterized by a common β subunit ($\beta 1$, $\beta 2$ and $\beta 3$) that can associate with one of a number of α subunits.

Recently, three further β subunits, $\beta 4$, βx (or βs) and βp, have been identified that form complexes with previously described α subunits (Sonnenberg *et al.*, 1988a; Cheresh *et al.*, 1989; Freed *et al.*, 1989; Hemler *et al.*, 1989; Holzmann *et al.*, 1989; Kajiji *et al.*, 1989).

The $\alpha 6$ subunit can associate with either the $\beta 1$ or the $\beta 4$ subunit. Complexes of $\alpha 6\beta 1$ (VLA-6) are found on platelets and on a variety of different epithelial cell types (Sonnenberg *et al.*, 1987, 1988a; Hemler *et al.*, 1988, 1989). The $\alpha 6\beta 1$ complex functions as a receptor for laminin (Sonnenberg *et al.*, 1988b). Recent studies have indicated that the site which is recognized by the $\alpha 6\beta 1$ complex is located on the E8 fragment of laminin (Sonnenberg *et al.*, manuscript submitted). The same laminin fragment has previously been reported to promote neurite outgrowth (Edgar *et al.*, 1984) and cell adhesion (Goodman *et al.*, 1987; Aumailley *et al.*, 1987). The ligand of $\alpha 6\beta 4$ is not known, but because cells expressing high levels of $\alpha 6\beta 4$ do not adhere to the E8 fragment of laminin, it is probably different from that of the $\alpha 6\beta 1$ complex.

Immunohistochemical analysis showed that $\beta 4$ expression is limited to epithelial cells and Schwann cells (Sonnenberg *et al.*, manuscript submitted). The $\beta 4$ subunit is particularly strongly expressed on squamous epithelial cells and is localized exclusively at the basal side of cells. This suggests a cell-matrix function for heterodimeric complexes of this subunit. A major difference between $\beta 4$ and all other β subunits described so far, is its much larger size (M_r 205 000 versus 90-130 000).

To study the relationship of $\beta 4$ with other β subunits and to determine the basis for the unusual size of $\beta 4$, detailed structural information was needed. In this report, we describe the isolation and sequence analysis of $\beta 4$ cDNA. We show that the $\beta 4$ subunit has an extracellular part that is homologous to other β subunits and a unique large cytoplasmic part. A search of the GenBank data base revealed no significant similarities of the cytoplasmic domain to other proteins.

Results

Isolation of $\beta 4$ cDNA

A lambda gt11 cDNA library prepared from poly(A)$^+$ RNA isolated from human keratinocytes was screened with the 439-9B monoclonal antibody against the integrin $\beta 4$ subunit (Kennel *et al.*, 1989). Screening of ~500 000 recombinants identified two positive clones; clone 134 contained an insert of 4.3 kb and clone 140 of 2.5 kb. Restriction fragment analysis showed that the insert of clone 140 was almost completely contained within clone 134 (Figure 1). The cDNAs hybridized to a single mRNA species of ~6 kb (Figure 2). This mRNA was present in the normal mammary cell line HBL-100 and the mammary tumor cell line T47D, which express the $\beta 4$ protein. Very low levels of $\beta 4$ mRNA, seen only on long exposed blots, were detected in OVCAR-4 and A375 cells and this is in accord with the low expression of the $\beta 4$ subunit in these lines. No mRNA was detected in the erythroid cell line K562. Both the distribution and the level of the 6 kb mRNA thus correspond to those of the $\beta 4$ protein. The two cDNAs were subsequently sequenced. The deduced amino acid sequence contained a cysteine-rich domain which was previously found to be conserved in the $\beta 1$, $\beta 2$ and $\beta 3$ subunits.

Overlapping cDNA clones that extended more to the 5' end of the $\beta 4$ cDNA were isolated by screening the same

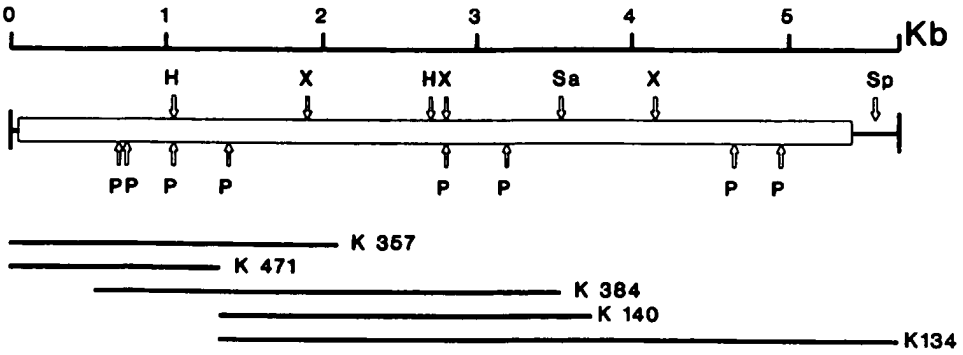


Fig. 1. Restriction map of the β4 cDNA clones. The open reading frame is shown as an open box. The lines indicate the size and order of the various cDNA clones. Relevant restriction sites are *Pst*I (P), *Xma*I (X), *Hin*dIII (H), *Sac*I (Sa) and *Sph*I (Sp).

cDNA library with a radiolabeled probe from the most 5′ portion of clone 140. An additional round of screening, using a 5′ fragment of the newly isolated clone 357 (see Figure 1) as a probe, did not result in cDNA clones extending further in the 5′ direction. The cDNA clones together spanned a stretch of ~5.7 kb. This size corresponds closely to the size of the mRNA detected in the Northern blot analysis (Figure 2).

β4 cDNA and deduced amino acid sequence

The cDNA sequence and the deduced amino acid sequence of β4 are shown in Figure 3. The nucleotide sequence of 5696 bp contains a small 9 bp 5′ untranslated leader followed by a single open reading frame of 5415 bp encoding polypeptide of 1805 amino acids and by a 3′ untranslated region of 272 bp. The 3′ untranslated region contains the polyadenylation signal AATAAA, followed 16 bases further by a poly(A) stretch.

The open reading frame starts with an ATG methionine codon that is flanked by sequences that meet the requirement for the initiation of protein translation (Kozak, 1984). Following the ATG methionine codon, there is a stretch of 27 mostly hydrophobic amino acids (−27 to −1) with the characteristics of a signal peptide (von Heijne, 1984). We assume, therefore, that the ATG codon represents the initiation site for translation, although no in-frame stop codon is observed in the 9-base sequence preceding this site. The deduced amino-terminal sequence of the mature protein matches, except at two positions, the amino-terminal amino acid sequence of purified β4 (Hemler *et al.*, 1989; Kajiji *et al.*, 1989). However, the amino acids at these two positions in the peptide sequence have not yet been unambiguously identified. Therefore, we conclude that the isolated cDNA is authentic for the β4 subunit.

The mature protein contains an extracellular region of 683 amino acids, a hydrophobic region of 23 amino acids and an exceptionally long cytoplasmic domain of 1072 amino acids. There are five potential N-linked glycosylation sites in the extracellular part of the β4 subunit. N-glycanase treatment of β4 resulted in a reduction of the size by ~10 000 dalton (Sonnenberg *et al.*, manuscript submitted). This may suggest that four of the five sites (average mol. wt per site is 2500 dalton) are glycosylated *in vivo*. The calculated mol. wt of the mature β4 subunit is 198 011 daltons; this value is close to the 195 000 daltons determined by SDS gel

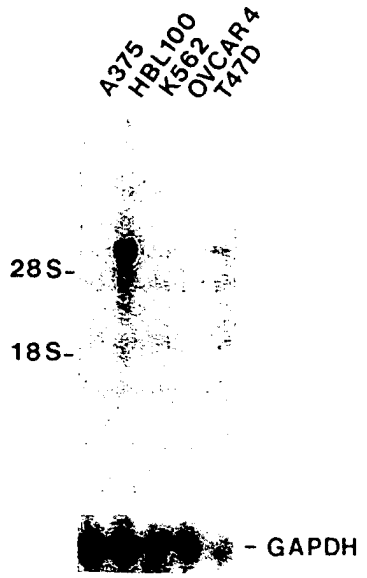


Fig. 2. Northern blot analysis. Total RNA (10 μg per lane) from A375, HBL100, K562, OVCAR-4 and T47D cell lines was electrophoretically separated on 1% agarose–formaldehyde gel, transferred to nitrocellulose filters and probed with β4 subunit cDNA (clone 134, 4.3 kb). In a control experiment (below) the same RNA samples were probed with glyceraldehydephosphatedehydrogenase cDNA.

electrophoresis for the deglycosylated β4 subunit. The extracellular portion of β4 has a relatively high cysteine content, 48 out of 683 amino acids (7%), in contrast to the cytoplasmic portion which only contains 15 cysteine residues (1.4%). Most of the cysteines of the extracellular portion of β4 are clustered at the amino terminus and in four homologously repeated cysteine-rich domains. Six of 15 cysteine residues in the cytoplasmic domain are located in a 15 amino acid stretch immediately following the transmembrane domain. These cysteine residues may be palmitoylated which may provide a better anchorage of the β4 subunit in the plasma membrane (Magee *et al.*, 1989).

Comparison with β subunits and other proteins

Figure 4 shows the alignment of the deduced amino acid sequence of β4 with the β1, β2 and β3 sequences. The overall identity between the extracellular part of the mature

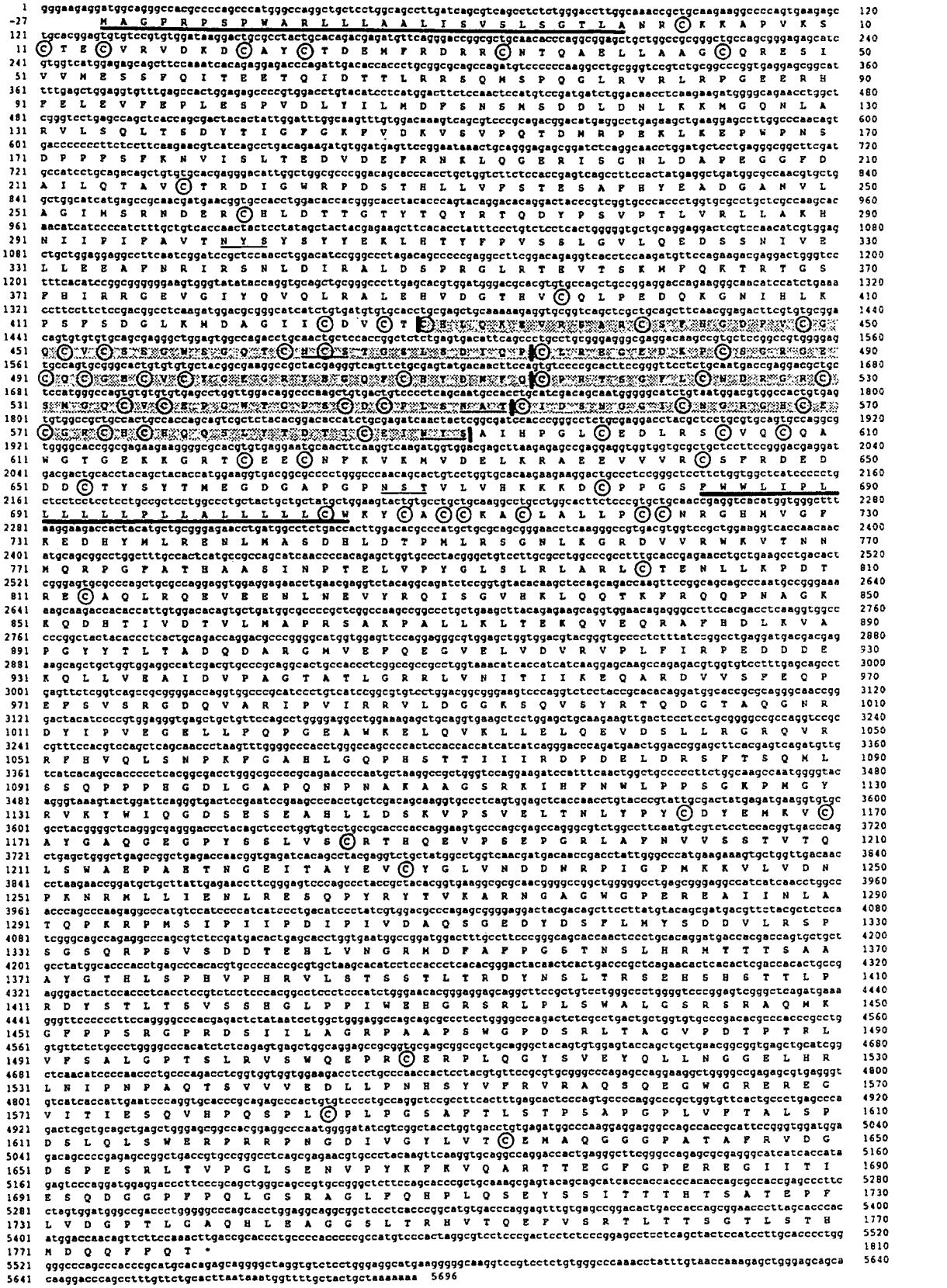


Fig. 3. Nucleotide sequence and the deduced amino acid sequence of β4 cDNA. The positions of the signal peptide (double underlined), the transmembrane region (heavily underlined) and the putative N-linked glycosylation sites (thinly underlined) are marked. The cysteine-rich domains are indicated with a shaded background and cysteine residues are circled.

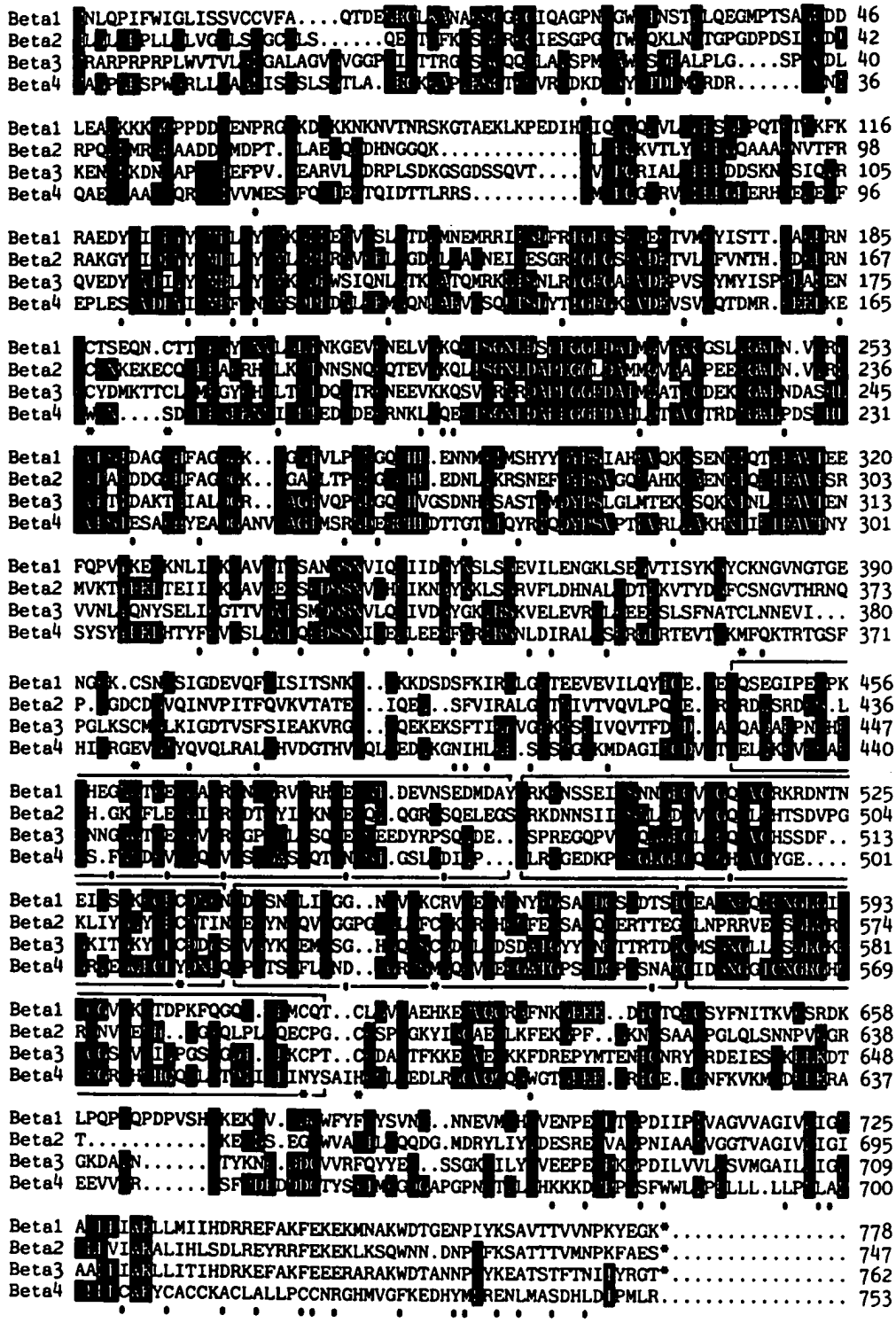


Fig. 4. Alignment of the β4 protein sequence with integrins β subunits. The β4 sequence is compared to three human β subunit sequences; β1 (Argraves *et al.*, 1987), β2 (Kishimoto *et al.*, 1987; Law *et al.*, 1987) and β3 (Fitzgerald *et al.*, 1987). Amino acids identical between the β4 subunit and any of the other three β subunits are indicated by inverse printing. Except for cysteine residues, all residues which are conserved in β1, β2 and β3 but not in β4 are indicated by solid circles. The cysteines lacking in β4 are marked with an asterisk and the cysteine-rich domains are boxed. The major part of the cytoplasmic domain of β4 has been omitted, because there is no corresponding sequence in β1, β2 or β3.

β4 subunit and each of the three other β subunits is ~35%. Identities in the order of ~45% were found in two long stretches of amino acids, one of 269 residues from positions 73 to 342 and another of 183 residues from positions 425 to 608. The latter region corresponds closely to the four homologous cysteine-rich repeat sequences. Between these two long homologous regions, there is a region of 83 amino acids with almost no identity. Of the 15 amino acids that are shared between all three β1, β2 and β3 subunits, only two are conserved in the β4 sequence. The first 72 amino

acids of the amino terminus and the 76 amino acids between the last cysteine-rich repeat sequence and the transmembrane domain are weakly conserved (20–30%) in β4. Also, the transmembrane region of β4 (residues 684–706) is weakly conserved, whereas the cytoplasmic portion of β4 is entirely unique.

Although the extracellular part of the β4 subunit is clearly similar to that of the three other β subunit sequences, this similarity is less than that between these three β subunit sequences (~35 versus ~45%). Furthermore, the β4 subunit lacks eight of the 56 cysteine residues that are conserved in all other three β subunit sequences. The positions of these residues are marked with an asterisk. Three of them are located in the cysteine-rich domains.

Computer searches of protein data bases revealed no significant similarities of the β4 subunit to proteins other than the β1, β2 and β3 subunits. Nevertheless, at the nucleotide level, a large region of β4 seems to be homologous with the non-muscle myosin heavy-chain gene of *Acanthamoeba* (Hammer *et al.*, 1987), i.e. 50% identity with multiple gaps in a stretch of 1400 nucleotides. This percentage is large, considering the evolutionary distance between the two species. However, because this homology is not found at the amino acid level, its significance is not clear. Probably, the fact that both regions are highly G/C-rich may account for the nucleotide homology of these two sequences.

In conclusion, it seems that the cytoplasmic domain of β4 is unique, not only in size but also in amino acid sequence.

Discussion

We here report the primary structure of β4, one of the newly characterized integrin β subunits. There are several observations that indicate that the sequence presented is authentic.

First, it is in accord with the amino-terminal sequence of the β4 subunit as published by Kajiji *et al.* (1989) and Hemler *et al.* (1989). Second, the RNA hybridization is consistent with the expression of β4 on cell lines. Third, the protein sequence has typical integrin β subunit-like structures. Finally, the predicted mol. wt of the polypeptide chain of 198 011 daltons agrees with the value determined after SDS–PAGE analysis of the deglycosylated β4 protein (Sonnenberg *et al.*, manuscript submitted).

Previously, Kajiji *et al.* (1989) have suggested that the larger size of β4 is due to the presence of a large amount of sialic acid, but from our present results it seems that the high mol. wt value of β4 is due to a very large cytoplasmic part comprising 1072 amino acids (~118 kd). In addition to the large cytoplasmic part, there are other distinctive features of the β4 subunit. The amino acid sequence of the extracellular part of β4 comprises the smallest number of conserved amino acids of all β subunits. In this regard, the β4 subunit lacks eight of the 56 cysteine residues, conserved in the three other human β subunits and in β1 sequences from the mouse (Holers *et al.*, 1989), chicken (Tamkun *et al.*, 1986) and *Xenopus laevis* (DeSimone and Hynes, 1988) and in the *Drosophila melanogaster* integrin β subunit, absent in *lethal myospheroid* mutants (Mackrell *et al.*, 1988). As the tertiary structure of β subunits is thought to be determined by intrachain disulfide bonds, the absence of these residues in the β4 subunit may have important structural and functional implications. Furthermore, a region of 63 amino acids, from position 109 to 171 in β3 implicated in RGD-mediated adhesion (D'Souza *et al.*, 1988), is strongly conserved in the β1, β2 and β3, but not in the β4 sequence. Only 28 of 48 amino acids match the conserved sequence for this region. Finally, in one of the least conserved regions (residues 324–425) β1, β2 and β3 share 15 amino acids, only two of which are present in the sequence of β4.

A further distinctive feature of β4 is its susceptibility to proteolytic degradation (Hemler *et al.*, 1989). The molecule can be degraded stepwise from a 205 via a 183 to a 150 kd product. Because the amino-terminal sequence of the 150 kd product matches the deduced sequence of the 205 kd mature protein, it can be concluded that both the 183 and 150 kd products have shorter versions of the cytoplasmic domain. A search in the cytoplasmic domain for endopeptidase cleavage sites, which usually consist of two or more basic residues, yielded eight of such sites. Cleavage at three of these sites may generate the β4 degradation products of 183 or 150 kd. They are located at positions 1621, 1294 and 1244. Recently, it has been shown that only the 205 kd band, but not the proteolytic degradation products of 183 and 150 kd, is phosphorylated (Kennel *et al.*, 1989). Therefore, the phosphorylation sites are assumed to be located within the last 20 kd region of the carboxy terminus. Indeed, there are several potential phosphorylation sites in this region for both serines and threonines. A tyrosine residue (residue 1668) might also be phosphorylated, because there is homology between the region around it and the phosphorylation site of β1 (residue 763) and other tyrosine kinase acceptor sites (Tamkun *et al.*, 1986).

Because the β1 and β4 but not the other β subunits associate with the α6 subunit, we searched for amino acids common to the sequences of only the first two. In the aligned sequences, 36 amino acids were found to be exclusively shared by the β1 and β4 subunits. A region immediately following the last cysteine-rich domain (residues 605–624) contained six of these exclusively shared amino acids, three of which were consecutive, i.e. EKK. This EKK sequence is also found on the β1 subunit of the mouse (Holers *et al.*, 1989) and chicken (Tamkun *et al.*, 1986). It is known that α6β1 complexes occur in the mouse (Sonnenberg *et al.*, 1988a) but not whether they also occur in chicken. The sequence is absent from the β1 subunit from *Xenopus laevis* (DeSimone *et al.*, 1988) and the β subunit from *Drosophila melanogaster* (Mackrell *et al.*, 1988), but whether these species possess α6β1 is unknown.

Integrins interact via their cytoplasmic domains with several other proteins to form transmembrane connections with the actin-cytoskeleton. These linker molecules include talin (Horwitz *et al.*, 1986), vinculin (Burridge and Mangeat, 1984), α-actinin (Chen and Singer, 1982) and the newly detected cytoplasmic protein, fibulin (Argraves *et al.*, 1989). It is tempting to speculate that one of the functions of the cytoplasmic domain of β4 is to interact directly with the cytoskeleton without the involvement of these linker proteins. We found no similarity of the β4 subunit to chicken α-actinin and vinculin. Unfortunately, the sequence of talin is not known and therefore, a direct comparison was not possible. Of the sequence of fibulin, only 15 amino acids of the amino terminus are known. Four of these amino acids, three of which are consecutive (LLE), are identical to amino acids in the sequence of the cytoplasmic part of β4 (residues

1032–1046). Therefore, there is at the moment no argument that part of the cytoplasmic tail of β4 is similar to linker proteins.

Materials and methods

cDNA cloning

The human keratinocyte lambda gt11 cDNA library (Clontech, Palo Alto, CA) was screened with the 439-9B rat monoclonal antibody against β4 (Kennel *et al.*, 1989) using the protoblot immunoscreening system of Promega. Clones that expressed insert-encoded protein reactive with the 439-9B antibody were isolated and plaque purified. The lambda DNAs were prepared by Lambda sorb immunoprecipitation (Promega Biotec) and the *Eco*RI inserts were subcloned into pUC18 or pUC19 for restriction mapping (Maniatis *et al.*, 1982). Additional screenings were done with radiolabeled DNA fragments from the 5′ portions of the previously isolated cDNA inserts as probes. DNA fragments were ^{32}P-labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1984).

Sequence analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) and 5′α [^{35}S]dATP as radioactive nucleotide. Sequences were obtained from one end of subcloned restriction fragments by using universal M13/pUC-sequencing primers. The internal sequence of large fragments was obtained from oligonucleotide primers that were synthesized on the basis of preceding sequence. Sequencing artefacts were avoided by determining all sequences using both dGTP and the nucleotide analogue deoxyinosine triphosphate (dITP) and by sequencing both strands of the cDNA insert.

Northern blot analysis

Total cellular RNA was isolated by the ureum/lithium chloride method (Barlow *et al.*, 1963) from cell lines which express high levels (HBL-100 and T47D), low levels (OVCAR-4 and A375) and no (K562) β4 protein. RNA was electrophoresed in a 1% agarose gel containing formaldehyde and transferred to nitrocellulose by standard procedures (Maniatis *et al.*, 1982). Hybridizations were carried out for 16 h at 64°C with ^{32}P-labeled insert probes in 3 × SSC containing 10% dextran sulphate, 5 × Denhardt's solution (1 × = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) and 50 μg/ml salmon sperm DNA. The filters were washed once with 3 × SSC, 0.1% SDS for 30 min, once with 1 × SSC, 0.1% for 30 min and twice with 0.1 × SSC, 0.1% SDS for 30 min at 64°C, dried and exposed to Kodak XAR-5 film with Dupont Cronex Lightning-Plus intensifying screens.

Data handling and analysis

Sequence data were analysed using GCG software (Devereux *et al.*, 1984). Nucleotide sequence and deduced amino acid sequences were used to search for homology in the Genbank (release 60.0).

Acknowledgements

We are grateful to Dr Stephen Kennel who supplied us with the anti-β4 antibody 439-9B; Drs Theo Cuypers, Hans Vos, Jan van Mourik and Paul Engelfriet for their contributions to this project and their helpful comments on the manuscript, and Wanda Winkel and Jetty Gerritsen for typing the manuscript. This work was supported by a grant from the Foundation for Medical Research MEDIGON, which is subsidized by the Netherlands Organization for Scientific Research (NWO) (grant no. 900-526-106).

References

Argraves,W.S., Suzuki,S., Arai,H., Thompson,K., Pierschbacher,M.D. and Ruoslahti,E. (1987) *J. Cell Biol.*, **105**, 1183–1190.

Argraves,W.S., Dickerson,K., Burgess,W.H. and Ruoslahti,E. (1989) *Cell*, **58**, 623–629.

Aumailley,M., Nurcombe,V., Edgar,D., Paulsson,M. and Timpl,R. (1987) *J. Biol. Chem.*, **262**, 11532–11538.

Barlow,J.J., Mathias,A.P., Williamson,R. and Gammack,D.B. (1963) *Biochem. Biophys. Res. Commun.*, **13**, 61–66.

Burridge,K. and Mangeat,P. (1984) *Nature*, **308**, 744–746.

Chen,W.-T. and Singer,S.J. (1982) *J. Cell Biol.*, **95**, 205–222.

Cheresh,D.A., Smith,J.W., Cooper,H.M. and Quaranta,V. (1989) *Cell*, **57**, 56–69.

DeSimone,D.W. and Hynes,R.O. (1988) *J. Biol. Chem.*, **263**, 5333–5340.

Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.

Edgar,D., Timpl,R. and Thoenen,H. (1984) *EMBO J.*, **3**, 1463–1468.

Feinberg,A.P. and Vogelstein,B. (1984) *Anal. Biochem.*, **132**, 6–13.

Fitzgerald,L.A., Steiner,B., Rall,S.C.Jr., Lo,S.-S. and Phillips,D.R. (1987) *J. Biol. Chem.*, **262**, 3936–3939.

Freed,E., Gailit,J., van der Geer,P., Ruoslahti,E. and Hunter,T. (1989) *EMBO J.*, **8**, 2955–2965.

Ginsberg,M.H., Loftus,J.C. and Plow,E.F. (1988) *Thrombos. Hemostas.*, **59**, 1–6.

Goodman,S.L., Deutzmann,R. and von der Mark,K. (1987) *J. Cell Biol.*, **105**, 589–598.

Hammer,J.A., III, Bowers,B., Paterson,B.M. and Korn,E.D. (1987) *J. Cell Biol.*, **105**, 913–925.

Hemler,M.E., Huang,C. and Schwarz,L. (1987) *J. Biol. Chem.*, **262**, 3300–3309.

Hemler,M.E., Crouse,C., Takada,Y. and Sonnenberg,A. (1988) *J. Biol. Chem.*, **263**, 7660–7665.

Hemler,M.E., Crouse,C. and Sonnenberg,A. (1989) *J. Biol Chem.*, **264**, 6529–6535.

Holers,V.M., Ruff,T.G., Parks,D.L., McDonald,J.A., Ballard,L.L. and Brown,E.J. (1989) *J. Exp. Med.*, **169**, 1589–1605.

Holzmann,B., McIntyre,B.W. and Weissman,I.L. (1989) *Cell*, **56**, 37–46.

Horwitz,A., Duggan,K., Buck,C., Beckerle,Mc. and Burridge,K. (1986) *Nature*, **320**, 531–533.

Hynes,R.O. (1987) *Cell*, **48**, 549–554.

Kajiji,S., Tamura,R.N. and Quaranta,V. (1989) *EMBO J.*, **8**, 673–680.

Kennel,S.J., Foote,L.J., Falcioni,R., Sonnenberg,A. Stringer,C.D., Crouse,C. and Hemler,M.E. (1989) *J. Biol. Chem.*, **264**, 15515–15521.

Kishimoto,T.K., O'Connor,K., Lee,A., Roberts,T.M. and Springer,T.A. (1987) *Cell*, **48**, 681–690.

Kozak,M. (1984) *Nucleic Acids Res.*, **12**, 857–872.

Law,S.K.A., Gagnon,J., Hildreth,J.E.K., Well,C.E., Willis,A.C. and Wong,A.J. (1987) *EMBO J.*, **6**, 915–919.

Mackrell,A.J., Blumberg,B., Haynes,S.R. and Fester,J.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2633–2637.

Magee,A.I., Gutierrez,L., Marshall,C.J. and Hancock,J.F. (1989) *J. Cell Sci.*, **11**, 149–160.

Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Ruoslahti,E. and Pierschbacher,M.D. (1987) *Science*, **238**, 491–497.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5466.

Sonnenberg,A., Janssen,H., Hogervorst,F., Calafat,J. and Hilgers,J. (1987) *J. Biol. Chem.*, **262**, 10376–10383.

Sonnenberg,A., Hogervorst,F., Osterop,A. and Veltman,F.E.M. (1988a) *J. Biol. Chem.*, **263**, 14030–14038.

Sonnenberg,A., Modderman,P.W. and Hogervorst,F. (1988b) *Nature*, **336**, 487–489.

D'Souza,S.E., Ginsberg,M.H., Burke,T.A., Lam,S.C.-T. and Plow,E.F. (1988) *Science*, **242**, 91–93.

Springer,T.A., Dustin,M.L., Kishimoto,T.K. and Marlin,S.D. (1987) *Annu. Rev. Immunol.*, **5**, 223–252.

Tamkun,J.W., Desimone,D.W., Fonda,D., Patel,R.S., Buck,C., Horwitz,A.F. and Hynes,R.O. (1986) *Cell*, **46**, 271–282.

von Heijne,G. (1984) *J. Mol. Biol.*, **173**, 243–251.

Received on December 11, 1989

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1990 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 265, No. 20, Issue of July 15, pp. 11502-11507, 1990
Printed in U.S.A.

Complete Amino Acid Sequence of a Novel Integrin β Subunit (β_6) Identified in Epithelial Cells Using the Polymerase Chain Reaction*

(Received for publication, February 23, 1990)

Dean Sheppard‡§, Carla Rozzo¶, Lisa Starr¶, Vito Quaranta¶, David J. Erle‡‖, and Robert Pytela‡**

*From the ‡Lung Biology Center, the Cardiovascular Research Institute, and the Departments of Medicine and **Pharmacology, University of California, San Francisco, California 94143 and the ¶Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037*

The integrin family of adhesion receptors consists of several heterodimeric glycoproteins, each composed of one α and one β subunit. Three different mammalian β subunits, β_1, β_2, and β_3, have been sequenced, but recent evidence suggests the existence of several others. Amplification of guinea pig airway epithelial cell cDNA with oligonucleotide primers designed to recognize consensus integrin β subunit sequences led to the identification of a novel partial cDNA sequence. Clones containing portions of this sequence were used to screen cDNA libraries constructed from the human pancreatic carcinoma cell line FG-2 and identified a series of overlapping clones encoding the full-length sequence of the human homologue of this protein. This sequence of 788 amino acids is 43, 38, and 47% identical to the sequences of β_1, β_2, and β_3, respectively. Features shared between this novel protein and the previously sequenced β subunits include the positions of all 56 cysteine residues in the extracellular domain, the single putative transmembrane domain, and the short putative cytoplasmic domain. However, a unique 11-amino acid extension at the carboxyl terminus, not present in any of the other β subunits, is suggestive of distinctive interactions with cytoplasmic components. Comparison of the human and guinea pig sequences reveals a high degree (94%) of cross-species conservation. Because this protein is clearly distinct from the two other recently described integrins β_4 and β_5, we propose to designate it β_6.

Integrins are a large family of cell surface glycoproteins that mediate cell to cell and cell to matrix adhesion (1, 2). All known members of this family are heterodimers consisting of an α and a β subunit that are noncovalently bound to each other. When the integrin family was first identified, integrins were grouped into three subfamilies based on the three β subunits that were initially recognized (β_1, β_2, and β_3). Each α subunit was thought to associate uniquely with a single β subunit. As new integrins have been identified, however, it has become clear that this grouping is not entirely satisfactory, since there are clearly more than three β subunits and since some α subunits can associate with more than one β subunit (3–8). Over the past few years, the primary structures of three integrin β subunits from mammalian cells and one from *Drosophila* have been deduced from cDNA (9–14). Alignment of these sequences reveals striking evolutionary conservation. Conserved structural features include the relative positions of all 56 cysteine residues as well as the length and sequence of the putative transmembrane and cytoplasmic domains. The most striking sequence conservation, including several short stretches of invariant amino acid residues, is observed in a large region in the amino-terminal half of the polypeptide. This region has been implicated in ligand binding by cross-linking studies employing peptide fragments of protein ligands which contain the integrin recognition sequence Arg-Gly-Asp (15, 16). Since this region appears to be extremely important for the functional integrity of the β subunit, the high degree of sequence conservation is likely to be shared by any additional members of the β subunit family. We used degenerate oligonucleotide mixtures designed to recognize consensus sequences in this region as PCR[1] primers to amplify both known and novel integrin β subunits. In this report, we present the complete deduced amino acid sequence of one novel β subunit identified in primary guinea pig airway epithelial cells and human pancreatic carcinoma cells.

MATERIALS AND METHODS

Generation of cDNA Fragments by PCR—Tracheal epithelial cells harvested from male Hartley outbred guinea pigs (Charles River Breeding Laboratories, Bar Harbor, ME) were grown to confluence over 10–14 days on collagen-impregnated microporous filters (Costar). RNA was harvested from primary cultures, and mRNA was purified over oligo(dT)-cellulose columns using the Fast Track mRNA isolation kit (Invitrogen). Two to 5 μg of mRNA was used as a template for cDNA synthesis catalyzed by 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in a 20–40-μl reaction volume. One to 5 μl of the resultant cDNA was used as a template for PCR. PCR was carried out in a reaction volume of 25–200 μl. In addition to the template cDNA, each PCR reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, and 0.05 units/μl *Taq* DNA polymerase (obtained from either United States Biochemical Corp. or from Promega). For each reaction, two oligonucleotide primers were also added to obtain a final concentration of 1 μM each. Each reaction mixture was overlaid with mineral oil, heated to 95 °C for 4 min in a thermal cycler (Ericomp, San Diego, CA), and then subjected to 30 cycles of PCR. Each cycle consisted of 45 s at 95 °C, 45 s at 53 °C, and 1 min at 72 °C. Immediately after the last cycle, the sample was maintained at 72 °C for 10 min.

* This work was supported in part by Research Grants HL/AI-33259 (to D. S.) and CA-47541 and CA-47858 (to V. O.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05522.

§ To whom reprint requests and correspondence should be addressed: Lung Biology Center, UC Box 0854, San Francisco, CA 94143. Tel.: 415-476-8580.

‖ Recipient of Training Grant HL 07155 from the National Institutes of Health.

[1] The abbreviation used is: PCR, polymerase chain reaction.

The results of each PCR reaction were analyzed by gel electrophoresis in 1.5% agarose. Reactions that produced fragments of the expected size were electrophoresed in 1.5% low gel temperature agarose (Bio-Rad). The appropriate size band was excised, melted at 68 °C, and the DNA was purified by extraction with phenol/chloroform and precipitation in ethanol and ammonium acetate.

PCR Primers—To obtain the initial fragment of the novel β subunit cDNA described in this report, degenerate mixtures of PCR primers were used. Oligonucleotides were synthesized by the UCSF Biomolecular Resource Center and purified over Nen-sorb cartridges (Du Pont-New England Nuclear). These consensus primer mixtures were designed to anneal with the nucleotides encoding the highly conserved sequence Asp-Leu-Tyr-Tyr-Leu-Met-Asp-Leu (primer B1F, Fig. 1) and Glu-Gly-Gly-Asp-Ala-Ile-Met-Gln (primer B2R) that flank an approximately 300-nucleotide region beginning approximately 130 amino acids from the amino terminus of each of the integrin β subunits sequenced to date (Fig. 1).

On the basis of the initial sequence obtained, we designed a specific forward primer to anneal with the sequence encoding the amino acids Pro-Leu-Thr-Asn-Asp-Ala-Glu-Arg (primer BTE2F, Fig. 1) ending approximately 49 nucleotides from the 3′ end of the region we had sequenced. We also designed an additional forward primer (B3F) and two reverse primers (B3R and B4R) to recognize highly conserved consensus regions encoding the sequences Gly-Glu-Cys-Val-Cys-Gly-Gln-Cys (B3 region) and Ile-Gly-Leu-Ala-Leu-Leu-Leu-Ile-Trp-Lys (B4 region). The alignment of these primers with previously published sequences of human β_1, β_2, and β_3 and chicken β_1 is shown in Fig. 1. We performed PCR as described above with cDNA from guinea pig tracheal epithelial cells and the primer pairs BTE2F/B3R and B3F/B4R.

The primer pair BTE2F/B3R yielded 1095 additional base pairs of new sequence. Based on this sequence another specific primer (BTE3F) was designed to recognize the sequence Val-Ser-Glu-Asp-Gly-Val near the 3′ end of this sequence, and PCR was performed with this primer in combination with primer B4R.

Cloning of Fragments Obtained by PCR—Individual fragments were cloned into pBluescript (Strategene) as follows. Purified fragments were resuspended in distilled water and treated with 2.5 units of DNA polymerase I, large (Klenow) fragment (Promega) to fill in any 3′ recessed ends left after the last cycle of PCR. The 5′ ends were phosphorylated with 5 units of T4 polynucleotide kinase (New England Biolabs). An aliquot of the above reaction mixture, containing approximately 100–200 ng of DNA, was ligated into pBluescript that had been cut with *Eco*RV (Promega) and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Ligations were performed at 22 °C for 1 h with T4 DNA ligase (Bethesda Research Laboratories). The ligation mixture was used to transform competent *Escherichia coli* (JM-109, Clontech). Plasmids containing inserts were purified using the Pharmacia miniprep lysis kit (Pharmacia LKB Biotechnology Inc.), denatured in 0.3 M NaOH, further purified over spinning columns containing Sephacryl S-400 (Pharmacia), and then sequenced using the Sequenase version 2.0 sequencing kit (United States Biochemical Corp.) and [^{35}S]dATP (Amersham Corp.).

Library Screening—PCR fragments generated with the primer pairs B1F/B2R and BTE3F/B4R were labeled with [^{32}P]dCTP and used as probes to screen a random-primed cDNA library and an oligo-dT-primed cDNA library, both constructed in the plasmid pTZ18R-BstXI (Invitrogen) from mRNA obtained from the human pancreatic carcinoma cell line FG-2. Plasmid was purified from clones found to hybridize with either region, and inserts were sequenced. A portion of insert DNA from one clone was in turn labeled and used to screen the same libraries. Fourteen independent overlapping clones were sequenced from both ends using primers that recognize regions of the pTZ polylinker. The regions flanking the 3′ end of the putative translated region of the new β subunit were sequenced in both directions from three clones using primers constructed to recognize sequences close to the 3′ end. On the basis of the initial sequences thus obtained, an additional internal sequence was obtained from clones T10, T11, T12, and T14 (Fig. 2) after digestion with specific restriction endonucleases and religation. Three internal fragments thus generated were subcloned into pBluescript and were also sequenced in both directions. Approximately 90% of the new sequence reported was obtained from both strands of DNA, and 97% was obtained from two or more overlapping clones (Fig. 2).

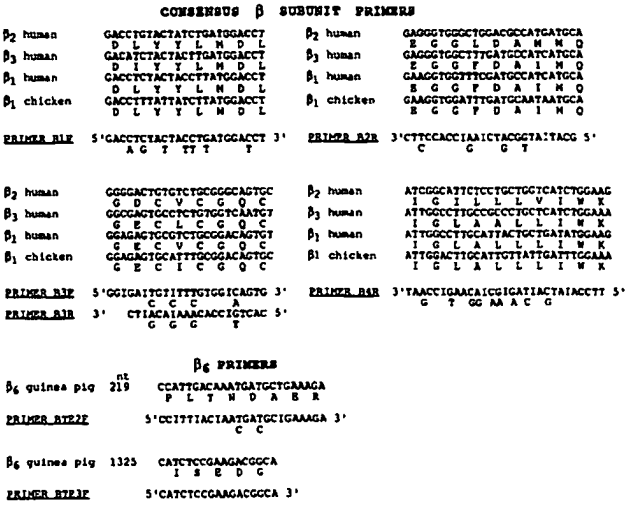


FIG. 1. **Design of PCR primers.** β subunit consensus primer mixtures were designed on the basis of alignment of published sequences of human β_1 (10), β_2 (11, 12), β_3 (13), and chicken β_1 (9). For forward primers (B1F and B3F), the primer sequences included a single nucleotide whenever possible for each of the first two nucleotides of each codon and were usually either degenerate or included deoxyinosine for the third base in codons for amino acids other than methionine. Reverse primers (B2R, B3R, and B4R) were designed in the same manner for the complementary DNA strand. Two specific forward primers were designed to recognize β_6. The first (BTE2F) was designed to work across species and was thus degenerate or included deoxyinosine in the third codon position. The second, BTE3F, was not degenerate and was designed to only recognize guinea pig β_6.

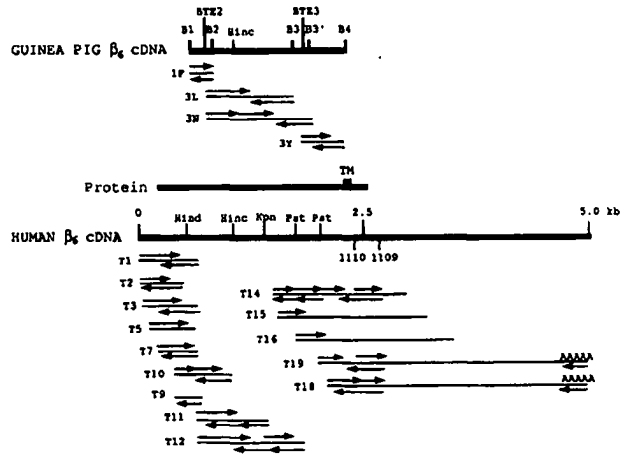


FIG. 2. **Map of sequencing strategy.** Shown are the location of clones used to obtain the partial cDNA sequence of guinea pig β_6 (clones *1F*, *3L*, *3N*, and *3Y*, *top*) and the complete sequence of human β_6 (clones *T1–T19*, *bottom*). Also, shown is the location of the translated region (*Protein*). The location of the transmembrane domain is shown by the letters *TM*. Clones shown often represent one of several identical clones. Internal sequence of clones with long inserts was obtained by restriction endonuclease digestion and religation and by ligation of internal fragments into pBluescript. Specific restriction sites employed are shown (*Hind*, *Hin*dIII; *Hinc*, *Hinc*II; *Kpn*, *Kpn*I; *Pst*, *Pst*I). The direction and extent of sequencing are shown by *arrows*. 1109 and 1110 are the sites recognized by oligonucleotide sequencing primers. T18 and T19 each terminated in a poly(A) tail. The regions recognized by the degenerate PCR primers B1F (*B1*), B2R (*B2*), B3R/F (*B3*), and B4R (*B4*) and the β_6 primers BTE2F (*BTE2*) and BTE3F (*BTE3*) are noted above the guinea pig cDNA map. *kb*, kilobases.

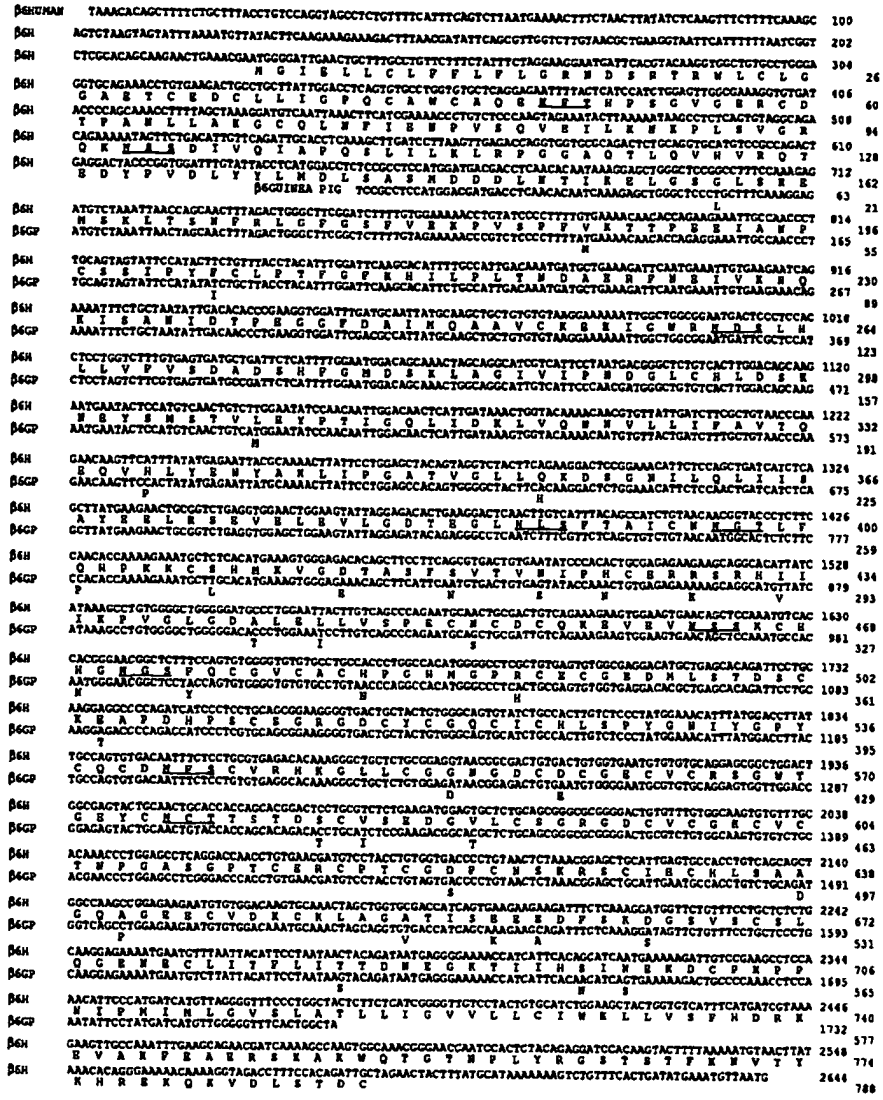


FIG. 3. **Nucleotide sequence and amino acid translation for human (*H*) and guinea pig (*GP*) β_6.** The amino acid translation is denoted by the single letter code beneath the second nucleotide of each codon from the translated region of human β_6. For the guinea pig sequence, only amino acids that differ from the human sequence are shown. The *numbers* along the *right-hand margin* denote the nucleotide or amino acid number of the last entry on each line. The numbering system used starts with the first nucleotide or amino acid available for each sequence shown. The nine potential sites for *N*-glycosylation in the putative extracellular domain of human β_6 are *underlined*.

RESULTS

Nucleotide Sequence of a Novel Guinea Pig Integrin β Subunit—PCR using cDNA from guinea pig airway epithelial cells and the consensus primer mixtures B1F and B2R (Fig. 1) amplified DNA fragments with the expected size of approximately 350 nucleotides. When the fragment DNA was sequenced after cloning into pBluescript, recombinant clones each contained inserts with one of two distinct sequences. One sequence encoded a stretch of 98 amino acids that was 97% identical to the expected region of human β_1 and was therefore presumed to be guinea pig β_1. The other sequence encoded 98 amino acids that were only 53% identical to human β_1, 45% identical to human β_2, and 57% identical to human β_3 (Fig. 2, clone *1F*). Both of the guinea pig sequences included the integrin β subunit consensus sequences Ser-*X*-Ser-Met-*X*-Asp-Asp-Leu and Gly-Phe-Gly-Ser-Phe-Val, and both contained the 2 cysteine residues found in this region in all known integrin β subunits. These data suggested that one of the two sequences we obtained encoded a new member of the integrin β subunit family.

This novel sequence was extended by further PCR steps utilizing primers specific for the novel sequence (BTE2F, BTE3F) in combination with two additional degenerate primers (B3R and B4R, see Figs. 1, 2, and 4). With the primer pair BTE2F/B3R two different cDNA products were obtained (*3L* and *3N* in Fig. 2) due to an unexpected hybridization of the B3R primer with a site 220 nucleotides further downstream (*B3′* in Fig. 2). The 1732-nucleotide sequence determined from these clones is shown in Fig. 3.

Nucleotide Sequence of Human β_6—Screening of cDNA libraries constructed from the human pancreatic carcinoma cell line FG-2 with guinea pig cDNA probes 1F and 3Y (see Fig. 2) and subsequent screening with a probe constructed from a portion of clone T10 (Fig. 2) produced 14 independent positive clones. The two longest clones (T18 and T19) extended to the poly(A) tail. A map of these clones, constructed on the basis of sequence information and of the mobility of inserts cut out of these clones in agarose gels is shown in Fig. 2. This map predicts an mRNA of approximately 5 kilobases including at least a 226-nucleotide untranslated region at the 5′ end, a 2364-nucleotide open reading frame, and a 3′-untranslated region of approximately 2.5 kilobases. For reasons discussed below, we have termed this molecule integrin β_6.

Fig. 3 shows the partial nucleotide and complete amino acid sequences for human β_6 (excluding most of the 3′-untranslated region) and the alignment of the 1732 nucleotides of sequence we obtained from PCR of guinea pig airway epithelial cell cDNA. Of the 577 amino acids deduced from the region we have sequenced in both species only 36 residues

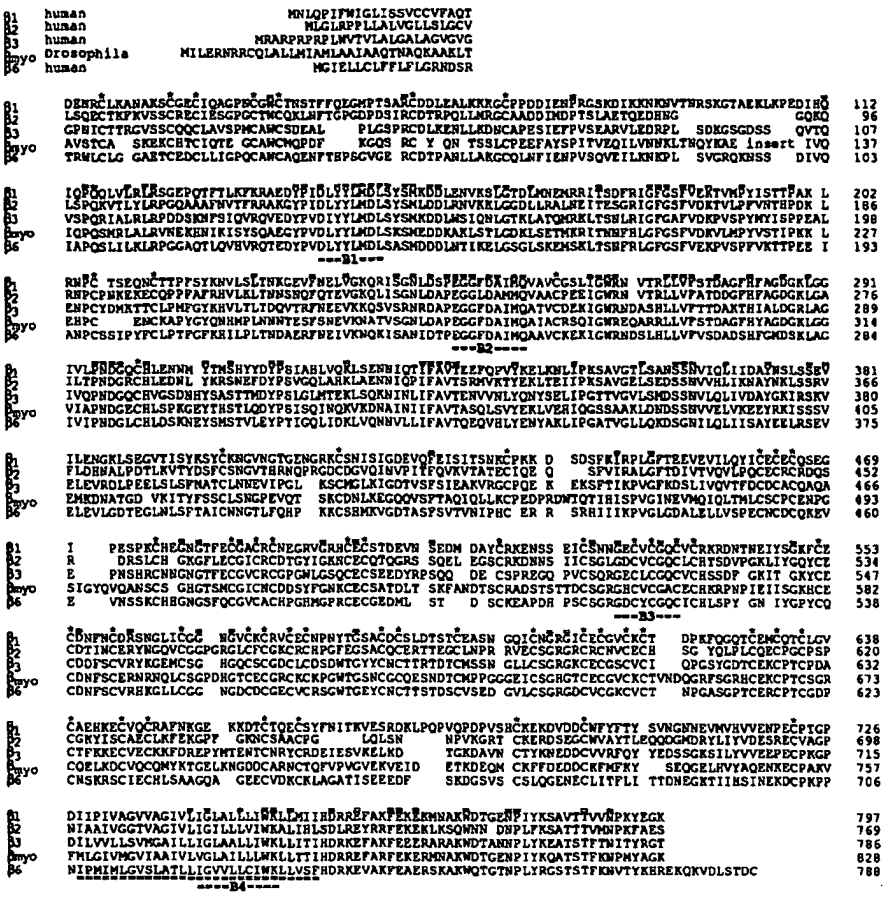


FIG. 4. **Alignment of β_6 with four previously reported integrin β subunits.** Previously published sequences for human β_1 (10), human β_2 (11, 12), human β_3 (13), the myospheroid gene product (β*myo*) of *Drosophila* (14), and the novel sequence we describe (β_6) are shown using the single letter amino acid code. The 56 conserved cysteines are noted by * and the 120 other invariant amino acids by = above each line. The transmembrane domain is *underlined*. The regions used for constructing the consensus β subunit primers B1F (*B1*), B2R (*B2*), B3F/R (*B3*), and B4R (*B4*) are labeled below the alignment in *bold type*. The *numbers* along the *right-hand margin* denote the number of the last amino acid in each line beginning from the first amino acid of each putative signal sequence.

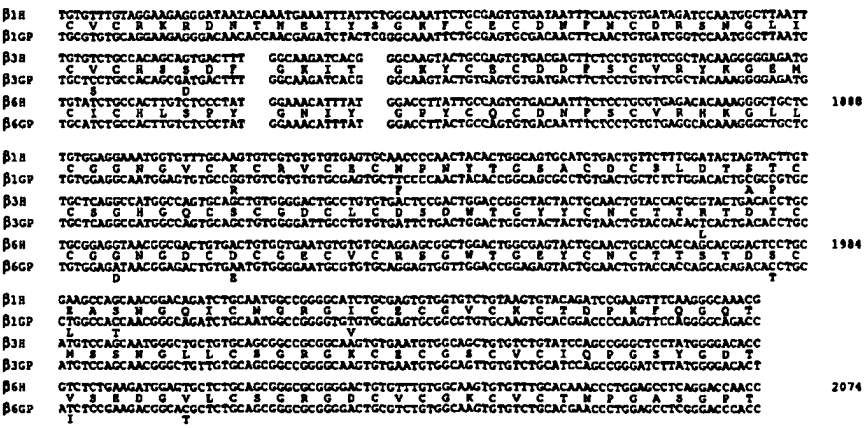


FIG. 5. **Alignment of partial nucleotide and amino acid sequences from human (*H*) and guinea pig (*GP*) β_1, β_3, and β_6 for the region just downstream from the B3F primer.** Amino acid translations denoted by the one-letter code are shown below the second nucleotide of each codon. For the guinea pig sequences, only amino acids that differ from the human sequences are shown. The *numbers* shown along the *right-hand margin* denote the nucleotide number for human β_6. The sequences for human β_1 (10) and β_3 (13) are from previously published reports.

differ; the amino acid sequences are 94% identical. Furthermore, of the 1732 nucleotides sequenced in both species, 91% are identical. Nine potential glycosylation sites present in the putative extracellular domain of human β_6 are shown by *underlining*. All seven of these sites that lie within the 577 amino acids obtained for guinea pig β_6 are also present in the guinea pig protein. If all of the potential glycosylation sites are occupied with oligosaccharides having an average molecular weight of 2,500, the predicted molecular weight of human β_6 would be 106,000.

Comparison of the 788-amino acid sequence deduced from the open reading frame to the three previously sequenced human β subunits and the myospheroid protein of *Drosophila* is shown in Fig. 4. There are 179 amino acid residues that are identical in each of the other β subunits and in β_6 including 56 conserved cysteine residues. The overall percentage of identical amino acids between β_6 and the other human β subunits is 47% for β_3, 42% for β_1, and 38% for β_2. Human β_6 is also 39% identical to the *Drosophila* β subunit. Human β_1, β_2, and β_3 and the *Drosophila* β subunit all have cytoplasmic regions consisting of 41 amino acids (beginning after the putative transmembrane domain shown by the *underline* in Fig. 4). Although β_6 contains each of the 10 conserved amino acid residues in this cytoplasmic region it also contains an 11-amino acid extension at the carboxyl terminus. β_6 also contains two Arg-Gly-Asp sequences, one at amino acids 514–516 and the other at 594–596. However, since these tripeptides are both within cysteine-rich regions, it is unclear whether they would be sufficiently exposed to serve as recognition sites for other members of the integrin family.

PCR using the primer pair B3F/B4R (see Fig. 1) amplified fragments of the expected size of approximately 750 nucleotides. Cloning and sequencing of the fragments did not result in any additional clones containing the novel β subunit sequence but did result in several clones with inserts encoding an amino acid sequence that was 97% identical to the corre-

sponding region of human β_3 and several others encoding an amino acid sequence that was 93% identical to human β_1 (Fig. 5). These are presumably the guinea pig homologues of β_1 and β_3, respectively. The nucleotide sequences of guinea pig and human β_1 are 80% identical, and those of guinea pig and human β_3 are 91% identical.

DISCUSSION

This report presents the complete amino acid sequence, deduced from cDNA, of a novel member of the human integrin β subunit family and a partial sequence of the guinea pig homologue of the same molecule. Comparison of this sequence to the previously published amino acid sequences of human β_1, β_2, and β_3 and to the myospheroid protein of *Drosophila* (Fig. 4) convincingly demonstrates that this protein is an integrin β subunit. There are 179 identical amino acid residues present in the novel protein we describe and in all of these other β subunits including all 56 conserved cysteine residues. As in each of the other integrin β subunits, the novel protein includes three complete repeats containing 8 cysteine residues each and one incomplete repeat (10–13). The amino acid sequence of this novel protein is 38–47% identical to each of the other β subunits, values quite similar to the degree of identity of β_1, β_2, and β_3 to one another. Like each of the other integrin β subunits, this novel protein is predicted to consist of a large extracellular domain with several potential glycosylation sites, a single transmembrane domain, and a short cytoplasmic tail. Comparison of the β chain sequences does not allow any direct insights into subunit association or ligand binding specificities. However, it should be noted that a stretch of nine amino acids at the NH_2-terminal end of a putative Arg-Gly-Asp-binding fragment is present in β_2, β_3, and β_6 but absent in β_2 (see Fig. 4, gap in β_2 sequence between residues 92 and 93). Since β_2 integrins do not bind extracellular matrix ligands in an Arg-Gly-Asp-inhibitable fashion, this deletion in the β_2 sequence may be related to its unique functional properties.

In addition to β_1, β_2, and β_3, recent studies have suggested the existence of as many as five other integrin β subunits. Some sequence information for two of these is available to us. A β subunit with a molecular weight of approximately 210,000 (β_4) has been found to associate with the integrin α subunit α_6 in colon carcinoma cells and in a variety of other tumor cells of epithelial origin (3, 6, 8). On the basis of its high molecular weight (210,000 compared with the predicted size of 106,000 of the novel protein we describe) and on the basis of its clearly different amino-terminal sequence, it is apparent that β_4 is not the same as the protein we describe. Another β subunit, originally called β_x, was identified in epithelial-derived tumor cells in association with the integrin α subunit α_v (4). The amino-terminal sequence of this β subunit, renamed β_5, has recently been determined from purified preparations and clearly differs from the β subunit we describe.[2] Because the β subunit described in the present report is distinct from each of the five β subunits for which sequence information is available, we have named it β_6.

The existence of two other integrin β subunits has been inferred from the identification of unique proteins after immunoprecipitation of surface-labeled cell lysates with antibodies to known α subunits. One of these novel proteins, called β_x, was found in association with α_v in the human osteosarcoma cell line MG-63, in the fibroblast cell line AF1523, and in human endothelial cells (5). This protein is clearly distinct from β_3, the β subunit that was first found to associate with α_v, on the basis of tryptic peptide mapping, lack of immunoreactivity with antibodies to β_3, and a unique pattern of phosphorylation in cells incubated with phorbol ester (5). Since no sequence information is available for β_x, we cannot be certain whether or not it is the same as β_6.

The other novel integrin β subunit identified by co-immunoprecipitation of known α subunits, β_p, is an M_r ~95,000 protein found in association with α_4, an α subunit first found as part of the lymphocyte homing receptor VLA-4 (7). The possible existence of one additional novel integrin β subunit can be inferred from evidence that there is a unique heterodimer on the surface of human neutrophils (17) that has ligand binding specificity for the tripeptide Arg-Gly-Asp, a sequence recognized by several members of the integrin family (1, 18). Since no other information about the sequence or cellular distribution of either of these β subunits is available, their relationship to β_6 is unclear.

A unique feature of the PCR-based technique we used to obtain the initial partial sequence of β_6 is the potential to rapidly obtain nucleotide sequences encoding similar proteins in more than one species. With this technique we obtained partial cDNA sequences that encode guinea pig β_1 and β_3 from the same cells (guinea pig airway epithelial cells) from which we obtained β_6. These data clearly show that at least these three β subunits are all present in these cells. Furthermore, they provide confirmation that the novel integrin β subunit sequence we obtained is not simply the guinea pig homologue of β_1 or β_3. Comparison of these sequences to the previously published sequences of human β_1 and β_3 confirms the remarkable cross-species conservation of the integrin β subunits, both for amino acids (93–97% identity) and nucleotides (80–91% identity).

An interesting feature of β_6 is its unique 11-amino acid carboxyl-terminal extension (Fig. 4). The short cytoplasmic tails of β_1, β_2, and β_3 have been suggested as important sites of interaction with the cytoskeleton and are probably critical regions for the transduction of signals initiated by interactions of the large extracellular domains with ligands. The cytoplasmic tails may also be important targets for regulation of integrin function. The unique extension of the cytoplasmic tail of β_6 implies that its regulation or its pathways for signal transduction may be different from those of β_1, β_2, and β_3.

Acknowledgment—We thank Theresa Kleven for help in the preparation of this manuscript.

REFERENCES

1. Ruoslahti, E. & Pierschbacher, M. D. (1987) *Science* **238**, 491–497
2. Hynes, R. O. (1987) *Cell* **48**, 549–554
3. Sonnenberg, A., Hogervorst, F., Osterop, A. & Veltman, F. E. M. (1988) *J. Biol. Chem.* **263**, 14030–14038
4. Cheresh, D. A., Smith, J. W., Cooper, H. M. & Quaranta, V. (1989) *Cell* **57**, 59–69
5. Freed, E., Gailit, J., van der Geer, P., Ruoslahti, E. & Hunter, T. (1989) *EMBO J.* **8**, 2955–2965
6. Hemler, M. E., Crouse, C. & Sonnenberg, A. (1989) *J. Biol. Chem.* **264**, 6529–6535
7. Holzmann, B., McIntyre, B. W. & Weissman, I. L. (1989) *Cell* **56**, 37–46
8. Kajiji, S., Tamura, R. N. & Quaranta, V. (1989) *EMBO J.* **8**, 673–680
9. Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F. & Hynes, R. O. (1986) *Cell* **46**, 271–282
10. Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D. & Ruoslahti, E. (1987) *J. Cell Biol.* **105**, 1183–1190
11. Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. & Springer, T. A. (1987) *Cell* **48**, 681–690
12. Law, S. K. A., Gagnon, J., Hildreth, J. E. K., Wells, C. E., Willis,

[2] D. Cheresh, personal communication.

A. C., and Wong, A. J. (1987) *EMBO J.* **6**, 915–919
13. Fitzgerald, L. A., Steiner, B., Rall, S. C., Jr., Lo, S. & Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 3936–3939
14. MacKrell, A. J., Blumberg, B., Haynes, S. R. & Fessler, J. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2633–2637
15. DSouza, S. E., Ginsberg, M. H., Burke, T. A., Lam, S. C. & Plow, E. F. (1988) *Science* **242**, 91–93
16. Smith, J. W. & Cheresh, D. A. (1988) *J. Biol. Chem.* **263**, 18726–18731
17. Gresham, H. D., Goodwin, J. L., Allen, P. M., Anderson, D. C. & Brown, E. J. (1989) *J. Cell Biol.* **108**, 1935–1943
18. Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell* **44**, 517–518

Novel Integrin α and β Subunit cDNAs Identified in Airway Epithelial Cells and Lung Leukocytes Using the Polymerase Chain Reaction

David J. Erle, Dean Sheppard, Johannes Breuss, Curzio Rüegg, and Robert Pytela

Lung Biology Center; Center for Occupational and Environmental Health; Cardiovascular Research Institute; Medical Service, San Francisco General Hospital; and the Departments of Medicine and Pharmacology, University of California at San Francisco, San Francisco, California

The integrins are a large group of cell surface glycoproteins that mediate cell-matrix and cell-cell adhesive interactions. Integrins play a role in normal lung development, in host defense against pulmonary infection, and in the pathogenesis of the adult respiratory distress syndrome. Integrins are heterodimers consisting of one α subunit and one β subunit. We identified consensus sequences within integrin subunits and used oligonucleotide primers based on these sequences to amplify cDNA by the polymerase chain reaction (PCR). We previously reported the use of this homology PCR technique for the identification of one novel integrin β subunit, β_6, from guinea pig airway epithelial cells. Here we demonstrate that primers based on α subunit consensus sequences can also be used for homology PCR. We have used the α and β subunit primers to amplify and clone a large variety of integrin partial cDNAs from several cell types and species. Comparison of the deduced amino acid sequences reveals a high degree of cross-species conservation (86 to 98% identity). One α subunit (identified in guinea pig airway epithelial cells) and one β subunit (identified in rabbit leukocytes obtained by bronchoalveolar lavage and in human and mouse leukocyte cell lines) have novel sequences that are related to but clearly distinct from all previously reported integrin sequences (24 to 61% identity). These novel cDNAs are very likely to encode previously unsequenced integrin subunit proteins that are expressed in the lung. Homology PCR is a powerful technique for the identification of known and novel integrin α and β subunit cDNAs in cells from the lung and other organs.

Cells adhere to other cells and to the extracellular matrix in a highly specific fashion during embryogenesis, inflammation, thrombosis, and metastasis. Many of these adhesive interactions are now known to be mediated by members of the integrin family of cell surface glycoproteins (1, 2). At least 16 mammalian integrins have been identified. All integrins are made up of one α subunit and one β subunit. Some, if not all, β subunits are capable of associating with more than one α subunit, and some α subunits are also able to associate with more than one β subunit. Both subunits are involved in determining ligand specificity. Integrins are expressed in a wide variety of lung cell types, including epithelial cells, endothelial cells, and leukocytes (3); the pattern of expression is developmentally regulated (4). Integrins on the surface of leukocytes are involved in the inflammatory response and in host defense against infection in the lung and other organs (5, 6). In animal models of the adult respiratory distress syndrome, lung injury can be dramatically attenuated by the administration of antibodies that block leukocyte integrin function (7–9). Integrins are also believed to play a role in the metastasis of some malignant cells to the lung (10).

The amino acid sequences of most of the known integrin α and β subunits have been determined (references cited in Tables 1 and 2). Eleven mammalian α subunit sequences have been previously reported. The overall structure of each of these α subunits is quite similar, and in several regions the amino acid sequence is highly conserved between many or all of the α subunits. The deduced amino acid sequences of five mammalian integrin β subunits, β_1, β_2, β_3, β_4, and β_5, have been previously reported by other investigators. These five sequences are 35 to 55% identical, if the unique large cytoplasmic portion of β_4 is excluded from the analysis. Integrin sequences are known to be highly conserved between species as diverse as human, chicken, and *Drosophila* (11–13). There are other α and β subunits that have been recently identified but that have not yet been sequenced. Examples of integrin subunits for which no sequence data have been reported include a novel melanoma laminin receptor α subunit (14), the β_7 subunit of lymphocytes (15, 16), and the α and β subunits of the "leukocyte response integrin" (17).

Our experiments were designed to obtain sequence infor-

(Received in original form September 26, 1990 and in revised form December 27, 1990)

Address correspondence to: David J. Erle, M.D., Lung Biology Center, Box 0854, University of California, San Francisco, CA 94143.

Abbreviations: bronchoalveolar lavage, BAL; base pairs, bp; polymerase chain reaction, PCR.

Am. J. Respir. Cell Mol. Biol. Vol. 5. pp. 170–177, 1991

mation about identified but unsequenced integrin subunits and to identify and sequence completely novel subunits. We were particularly interested in studying cells obtained directly from the lung. Homology polymerase chain reaction (PCR), the technique we employed in our experiments, has been used by others to identify novel members of several protein families (18–20). We previously reported the use of homology PCR for the identification of a novel β subunit, β_6, in guinea pig airway epithelial cells (21). We have now been able to extend this technique to the α subunit family. Here we report the use of homology PCR for the identification of known and novel cDNAs encoding members of both the α and the β subunit families in a wide variety of cell types of pulmonary and nonpulmonary origin.

Materials and Methods

Cells

Guinea pig airway epithelial cells were cultured in hormone-supplemented Ham's F-12 with vitamin A and 5% fetal bovine serum (22) on filter supports coated with types I and III collagen. Cells proliferated for 7 to 10 days, and RNA was obtained between days 10 and 15. Rabbit leukocytes (gift of Dr. A. Boylan, University of California, San Francisco, CA) were obtained by bronchoalveolar lavage (BAL) and stimulated with *Escherichia coli* lipopolysaccharide 10 μg/ml for 2 h prior to harvesting RNA from adherent cells (> 90% macrophages). Human cell lines used as sources of RNA were HeLa (cervical carcinoma), MeWo (melanoma), Raji (Burkitt's lymphoma), MoLT-4 (T-cell leukemia), and EBV-B6.1 (EBV-transformed peripheral B cells, gift of Dr. S. Abrignani, CIBA/Geigy, Basel, Switzerland). Mouse leukocyte lines used were WR 2.3 (lymphoma) and P388D1 (macrophage-like cells). Cell lines were grown in Dulbecco's modified Eagle's medium with Earle's balanced salt solution (MeWo) or in RPMI (all other cell lines) with 10% fetal or neonatal bovine serum on tissue culture plastic. Pig aortic endothelial cells (gift of Dr. J. Escobedo, University of California, San Francisco, CA) were from primary cell cultures (early passage).

cDNA Synthesis and PCR Amplification

Random hexadeoxynucleotide-primed cDNA was synthesized from 1 to 5 μg of poly(A)$^+$ RNA or 10 to 25 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (standard preparation or RNase H$^-$ preparation; Bethesda Research Laboratories, Gaithersburg, MD) in a 20- to 40-μl reaction. One to five microliters of the reverse transcription reaction were used in each PCR. PCR was done in *Taq* buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0] at 25° C, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100) with 0.1 mM each of dATP, dCTP, dGTP, and dTTP, approximately 1 μM of each primer mixture, and 2.5 U *Taq* DNA polymerase/100-μl reaction volume. Primer sequences were based on the sequence alignments shown in Figure 1. Restriction sites for Eco RI (GAATTC) or Sal I (GTCGAC) were incorporated into the 5′ ends of primers to facilitate cloning. Actual primer sequences were: A14F 5′-CGGAATTCGGIGA(A,G)CAG(A,C)TIG(C,G)I(G,T)CITA(C,T)TT(C,T)GG-3′, A2AR 5′-CTCGTCGACGGIGCI(C,G)CIA(C,T)IGCIA(C,T)(A,G)TCITI(A,G)(A,T)AICC(A,G)TC-3′, B1F 5′-CGTCGAC(A,C)T(C,G)TA(C,T)TA(C,T)(C,T)T(G,T)ATGGA(C,T)CT-3′, B2R 5′-TTTGAATTCATIAT(G,T)GC(A,G)TCIA(A,G)ICCACC(C,T)TC-3′, B1AF 5′-AAAGTCGACCCI(A,G)TIGA(C,T)(A,C,T)TITA(C,T)(A,T)(A,T)I(C,T)TIATGGA-3′, and B2AR 5′-GGGGAATTC(C,T)TGIA(A,G,T)IA(C,T)IGC(A,G)TCIA(A,G)ICCICC(C,T)TCIGG-3′, where I indicates deoxyinosine. Note that the sequences of the A2AR, B2R, and B2AR primers, which are intended to prime synthesis of the antisense strand, contain the reverse complement of the appropriate consensus sequence shown in Figure 1. The B1F and B2R primers were used in the initial experiments involving guinea pig, human, and mouse cDNA amplification; the B1AF and B2AR primers were used in later experiments with pig and rabbit cDNAs. Amplification was performed in a thermal cycler (Ericomp, San Diego, CA). For the α subunit amplifications, the reaction conditions were initial denaturation at 95° C for 4 min, 30 cycles of amplification (melting at 95° C for 45 s, annealing at 48° C for 45 s, extension at 72° C for 60 s), and final extension at 72° C for 10 min. For the β subunit amplifications, the reaction conditions were the same except that the annealing temperature was 53° C.

Cloning and Analysis of PCR Products

Amplified cDNA was prepared for cloning by phenol/chloroform extraction and ethanol precipitation followed by digestion with Eco RI and Sal I, which recognize sequences at the 5′ ends of the primers. The cDNAs were then purified by electrophoresis in low-melting-point agarose followed by phenol/chloroform extraction and ethanol precipitation. Amplified cDNAs were ligated into Eco RI- and Sal I-digested, dephosphorylated pBluescript vector (Stratagene, La Jolla, CA) with T4 DNA ligase. After transformation of competent JM109 *Escherichia coli*, up to 40 colonies were picked for further analysis. Clones were analyzed by DNA sequencing and/or restriction analysis. The sequences presented here were obtained by sequencing both strands of cloned cDNAs using modified T7 polymerase and the T3 and T7 primers. Sequence comparisons were performed with the GAP computer program (23), with gap weight set to 3.000 and length weight set to 0.100.

The "Restriction-Reamplification" Technique

We devised a technique we call "restriction-reamplification" to identify additional cDNAs in some cell types. We began by analyzing the one or two most abundant cDNA sequences determined from the clones obtained in the first round of amplification. Predicted restriction endonuclease sites within these sequences were identified. An aliquot of the amplified cDNA mixture was digested with one or two restriction endonucleases to digest the abundant cDNAs within the mixture. Less abundant cDNAs that did not contain a recognition site for the restriction endonuclease would presumably remain intact. In order to obtain sufficient material for cloning, the digested mixture was reamplified with the same consensus primers and reaction conditions used in the initial amplification. The resulting product was then cloned and analyzed as above. Restriction endonucleases used were: Pst I plus Pvu II (to digest guinea pig α_v plus α_5), Hinf I (to digest human β_1), and Rsa I (to digest mouse β_1).

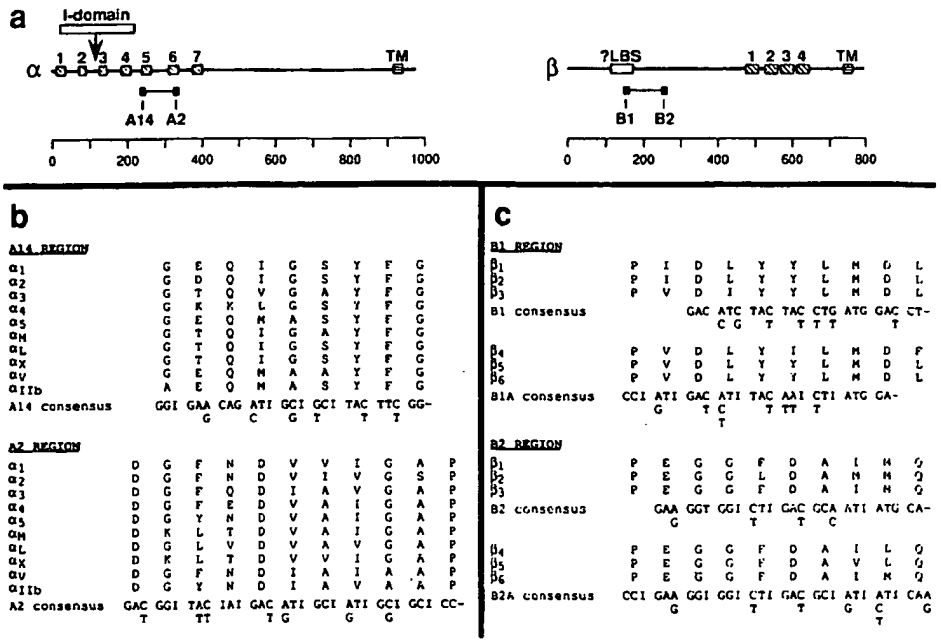


Figure 1. Alignments of conserved regions of the integrin α and β subunits used to design consensus polymerase chain reaction (PCR) primers. (a) Schematic diagram of the integrin α and β subunit structures. The α subunits contain large extracellular portions with seven repeated domains (labeled 1 through 7), a single transmembrane domain (TM), and short cytoplasmic portions. Five α subunits, α_1, α_2, α_M, α_L, α_X, have an additional domain (I-domain) not present in the other sequenced α subunits (25). The positions of the two conserved regions A14 and A2 are indicated by solid boxes. The β subunits contain large extracellular portions with four cysteine-rich regions (1 through 4), transmembrane domains (TM), and intracellular portions. The β_4 subunit, unlike the other β subunits, has an extremely large cytoplasmic portion (not shown). The two conserved regions B1 and B2 are near a putative ligand binding site (?LBS) identified by crosslinking studies (24). (b) α subunit alignments. Two highly conserved regions, A14 and A2, were identified by inspection of the previously reported human α_2, α_4, α_5, α_M, α_L, α_X, α_V, and α_{IIb}, rat α_1, and hamster α_3 subunit sequences (references cited in Table 1). Degenerate consensus nucleotide sequences that included most of the possible codon combinations that could encode these regions were generated and incorporated into the A14F and A2AR primer mixtures. Deoxyinosine (I) was incorporated at some positions where any of the four nucleotides might appear. In some cases where a marked codon preference was observed in the published cDNA sequences, only the preferred codon sequence was included in the consensus sequence. (c) β subunit alignments. The well-conserved B1 and B2 regions were identified by comparison of three previously reported human β subunit sequences, β_1, β_2, and β_3. The B1 and B2 consensus sequences were generated based on these three sequences. Later, when the β_4, β_5, and β_6 sequences became available to us, we added these sequences to the alignment and developed revised consensus sequences, designated B1AF and B2AR. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Results

Design of Consensus Oligonucleotide Primers

When we began our initial experiments, the sequences of three mammalian integrin β subunits, β_1, β_2, and β_3, had been reported. We aligned these three sequences and identified two regions, which we designated as B1 and B2, which were highly conserved (Figure 1). These regions are located in the extracellular portion of the β subunits near a putative ligand binding site (24) and are separated from each other by approximately 100 amino acid residues. We designed one oligonucleotide mixture (B1F) based on the B1 region, and a second mixture (B2R) based on the B2 region. After the sequences of β_4, β_5, and β_6 were reported, we revised our primer design based on these additional sequences (Figure 1c). The resulting primers, designated B1AF and B2AR, were used in the experiments involving rabbit leukocytes and pig aortic endothelium.

The previously reported α subunit sequences were also aligned and two highly conserved regions, located within the fifth and sixth repeated domains (25), were identified (Figure 1). These regions, designated A14 and A2A, are separated by between 72 and 92 amino acid residues in the previously reported α subunit sequences. The oligonucleotide primers A14F and A2AR were designed based on these regions.

PCR Amplification of Integrin cDNAs

We prepared cDNA by reverse transcription of RNA derived from a variety of cells (see MATERIALS AND METHODS). Cells from a total of five different species (human, guinea pig, mouse, pig, and rabbit) were studied. Cell types studied included leukocytes, epithelial cells, and endothelial cells. RNA was prepared from transformed cell lines, from cells in primary culture, and from cells obtained by BAL. Appropriate primer pairs (B1F/B2R, B1AF/B2AR, or A14F/A2AR) were used to amplify the cDNA. The PCR products were analyzed by agarose gel electrophoresis (Figure 2). When the α subunit primers were used, two bands corresponding to DNA products of approximately 350 and 290 base pairs (bp) were seen on agarose gel electrophoresis (lane 2). Based on the known α sequences, we expected that the amplified α_1 partial cDNA (including primers) would be approximately 350 bp in length and that the other partial cDNAs would be approximately 285 to 300 bp in length. When the β subunit primers were used, the PCR yielded products of approximately 360 bp (lane 3). This agrees with the size predicted from the known β subunit sequences.

PCR products were ligated into the pBluescript plasmid for cloning and further analysis. This resulted in the identification of between one and four different integrin subunit partial cDNAs per amplification (see below). We anticipated

Figure 2. Amplification of integrin α and β subunit cDNAs. Results shown here were obtained using pig aortic endothelial cells and are similar to the results obtained with other cells. RNA was prepared from cultured cells and reverse-transcribed to cDNA. The cDNA was amplified by PCR using integrin consensus primers and analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Lane 1: DNA size markers (ϕX 174 Hae III digest); bands, from top to bottom, correspond to fragments of 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118, and 72 base pairs. Lane 2: PCR products obtained with α subunit primers A1AF and A2AR. Lane 3: PCR products obtained with β subunit primers B1AF and B2AR.

that additional integrin cDNAs might not have been detected with this approach because of the small number of clones analyzed. This could occur if these cDNAs were less abundant or were amplified less efficiently than other integrin cDNAs. To address this problem, we used a modification of the PCR technique that we call "restriction-reamplification" (see MATERIALS AND METHODS). This technique did allow us to identify additional α or β subunit cDNAs in a number of cell types (see below).

α Subunit Sequences

The results of the α subunit amplification experiments are summarized in Table 1 and Figure 3. We used the α primers with cDNA made from guinea pig airway epithelial cells in primary culture, from human leukocytes (EBV-B6.1 cells) and from pig aortic endothelial cells. We were able to identify between two and six distinct α subunits in each cell type studied. A total of seven distinct α subunit cDNAs were identified in one or more species.

We were able to identify six different α subunit cDNAs present in guinea pig airway epithelial cells. The partial amino acid sequences deduced from five of these cDNAs are 88 to 92% identical to the sequences of α_1, α_2, α_3, α_5, and α_v from other species. These cDNAs presumably encode the guinea pig homologs of those five α subunits. The sixth sequence, provisionally designated as α_A, is 24 to 60% identical to the 11 previously reported α subunits in this region. The α_A partial nucleotide and deduced amino acid sequences are shown in Figure 4a. (Pairwise sequence comparisons were performed using the GAP computer program [23]; these pairwise alignments differ slightly from the subunit family alignment shown in Figure 3.) Short regions of the α_A sequence are very similar to corresponding regions of the other α subunits, especially α_4. Other regions are clearly distinct from α_4 and the other α subunits. These observations strongly suggest that α_A is a novel integrin α subunit that is distinct from the 11 other α subunits for which sequence data have been reported (see DISCUSSION). Two of the six guinea pig airway epithelial cell α subunits were identified by restriction-reamplification: neither α_2 nor α_3 cDNAs were identified among the 40 clones obtained with the basic method, but both α_2 and α_3 cDNAs were included in the five clones obtained by restriction-reamplification.

Our inability to clone the human α_L cDNA from EBV-B 6.1 cells may be related to the cloning strategy we used. The human α_L partial cDNA has a Sal I site very near the 5′ end (26). When the amplified cDNA mixture is prepared for cloning by digestion with Eco RI and Sal I, the α_L cDNAs would be digested into one relatively large piece with two Sal I ends, and one small piece that would be lost in the gel purification step. This would prevent the α_L cDNA from ligating with the Eco RI– and Sal I–digested vector, and no α_L clones would be obtained. This problem could presumably be overcome by modifying the cloning strategy.

β Subunit Sequences

The results of the β subunit amplification experiments are summarized in Table 2 and Figure 5. We used the β primers with cDNA made from lipopolysaccharide-stimulated rabbit BAL fluid leukocytes (> 90% macrophages), from human leukocyte cell lines (Raji and MoLT-4 lymphocytes), from mouse leukocyte cell lines (WR 2.3 lymphoma cells and P388D1 macrophage-like cells), from guinea pig airway epi-

TABLE 1
Summary of α subunit amplifications

α Subunit*	cDNA Source
α_1 human	EBV-B6.1 cells
α_1 rat (27)	–
α_1 guinea pig	Airway epithelial cells
α_1 pig	Aortic endothelial cells
α_2 human (28)	–
α_2 guinea pig	Airway epithelial cells†
α_3 hamster (29)	–
α_3 guinea pig	Airway epithelial cells†
α_3 pig	Aortic endothelial cells
α_6 human (30)	–
α_4 human (31)	EBV-B6.1 cells
α_A guinea pig	Airway epithelial cells
α_5 human (32)	–
α_5 guinea pig	Airway epithelial cells
α_5 pig	Aortic endothelial cells
α_v human (33)	–
α_v guinea pig	Airway epithelial cells
α_v pig	Aortic endothelial cells
α_{IIb} human (34)	–
α_M human (35, 36)	–
α_L human (26)	–
α_X human (37)	–

* The α subunit sequences are shown in Figure 3.
† Sequence was identified by the restriction-reamplification technique (see text).

Figure 3. Partial deduced amino acid sequences of integrin α subunits. Sequences shown include previously reported α subunit sequences and α subunit sequences determined from the experiments presented here (see Table 1). Gaps (−) have been introduced in order to maximize the alignment. Amino acid residues conserved in all α subunits are indicated by asterisks; residues conserved in all but one subunit are indicated by dots. Residues in rat, guinea pig, and pig sequences that differ from those in the human sequence are underlined. (In the case of α_3, where the human sequence of this region has not yet been reported, differences from the previously reported hamster sequence are underlined instead.) Abbreviations for the amino acids are given in the legend to Figure 1.

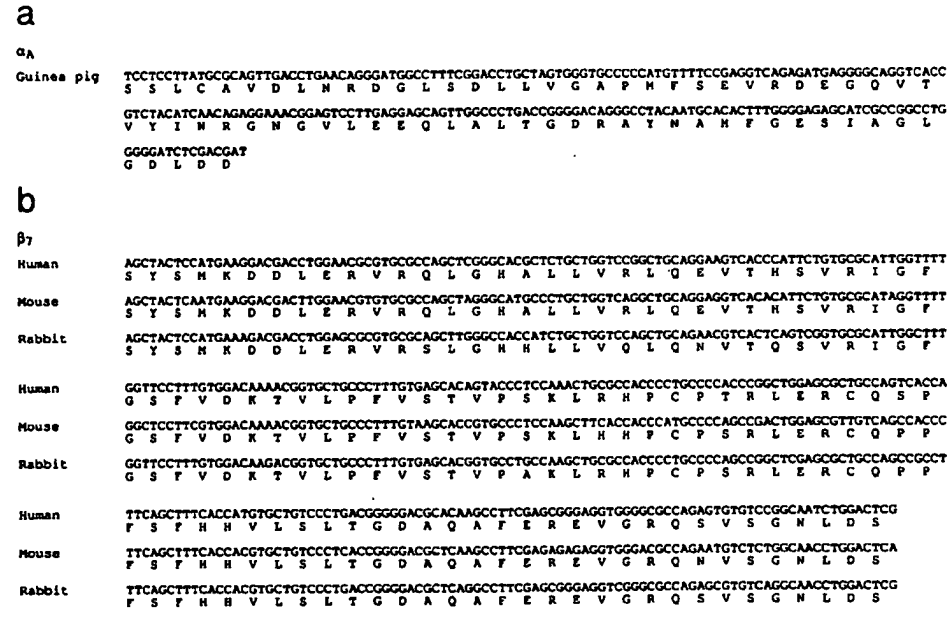


Figure 4. Partial nucleotide and deduced amino acid sequences of α_A and β_7. (a) Guinea pig α_A partial sequence. (b) Human, mouse, and rabbit β_7 partial sequences. Deduced amino acid sequences are shown below the first nucleotide of each codon. Abbreviations for the amino acids are given in the legend to Figure 1.

thelial cells, and from pig aortic endothelial cells. We were able to identify between one and three distinct β subunits in each cell type studied. A total of six distinct β subunit cDNAs were identified in one or more species.

We identified cDNAs encoding a novel integrin β subunit, designated β_7, in rabbit leukocytes obtained by BAL and in human and mouse leukocyte cell lines (Figure 4b). Amino acid sequences deduced from rabbit, human, and mouse β_7 cDNAs are 92 to 96% identical to one another. In contrast, the β_7 sequences are only 40 to 61% identical to other, previously reported integrin β subunits in this region. Short regions that are almost completely conserved in all previously reported integrin β subunit sequences are also conserved in each of the β_7 sequences. The positions of the two cysteine residues present in this portion of β_1, β_2, β_3, β_5, and β_6 (but not in β_4) are preserved in β_7. These striking similarities strongly suggest that the β_7 cDNAs encode human, mouse, and rabbit integrin β subunits for which no sequence data has previously been reported.

We were able to amplify cDNAs encoding β_1, β_2, β_3, β_5, and β_6. β_1 was amplified from all the cells tested. β_5 was not detected with the basic method (0/8 clones from MeWo cells, 0/8 clones from HeLa cells), but after restriction-reamplification was used to eliminate the abundant β_1 cDNAs, β_5 was easily detected (3/5 clones from MeWo cells, 1/1 clone from HeLa cells). Of note, these experiments were performed with the B1F and B2R primers, which were designed before the β_5 sequence was known to us. The B2R

TABLE 2
Summary of β subunit amplifications

β Subunit*	cDNA Source
β_1 human (32)	Raji, MoLT-4, HeLa, MeWo
β_1 mouse (38)	WR 2.3, P388D1
β_1 guinea pig (21)	Airway epithelial cells
β_1 pig	Aortic endothelial cells
β_2 human (39, 40)	Raji, MoLT-4
β_3 human (41)	MeWo
β_4 human (42, 43)	–
β_5 human (44, 45)	HeLa†, MeWo†
β_6 human (21)	–
β_6 guinea pig (21)	Airway epithelial cells
β_7 human	Raji, MoLT-4
β_7 mouse	WR 2.3, P388D1†
β_7 rabbit	Bronchoalveolar lavage cells

* The β subunit sequences are shown in Figure 5.
† Sequence was identified by the restriction-reamplification technique (see text).

primer has four mismatches with the reported β_5 sequence and may therefore amplify β_5 cDNA less efficiently than other β subunit cDNAs. We have previously reported the use of homology PCR to identify the guinea pig β_6 partial cDNA, and the use of the guinea pig β_6 cDNA clone to obtain the complete amino acid sequence of human β_6 (21).

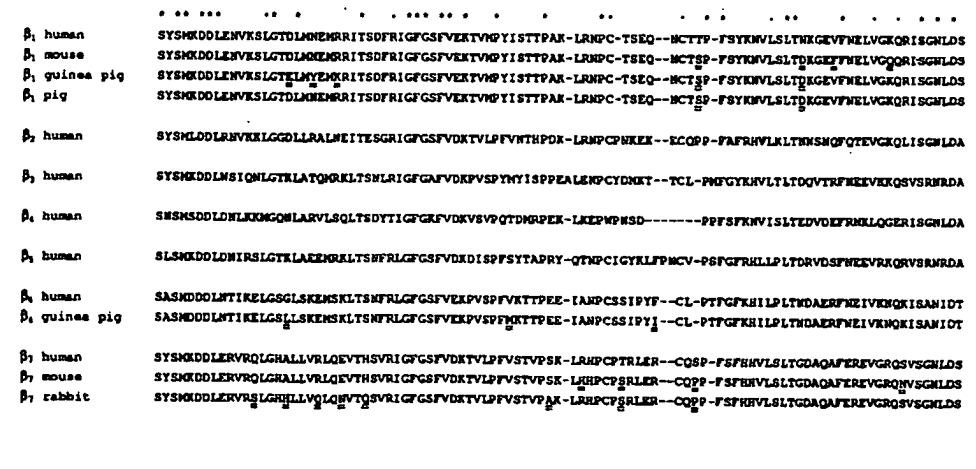
Discussion

We used the new technique of homology PCR to study the integrin family of adhesion molecules. The homology PCR technique has been successfully applied to other protein families (18–20), and, based on the high degree of sequence conservation between integrins, we anticipated that this technique could also be successfully applied to the integrin α and β subunit families. Alignments of the previously determined sequences of the β_1, β_2, and β_3 subunits revealed the existence of several extremely well-conserved regions. We designed a pair of primer mixtures based on two of these regions and used them to amplify guinea pig airway epithelial cell cDNA. These experiments, which have been previously reported (21), led to the identification of a novel integrin β subunit, β_6. Here we demonstrate that this technique can be successfully applied to a wide variety of cell types from different species, and we report the partial sequence of another novel integrin β subunit cDNA. We also report the extension of this technique to the integrin α subunits. Our initial attempts to amplify α subunits were much less successful: using 10 different primer pair combinations based on several regions of the α subunits which appeared to be conserved, we identified only a few known α subunit cDNAs from all of the cell types we studied (data not shown). As additional α subunit sequences were reported in the literature, it became clear that the number of regions that are highly conserved between all or virtually all α subunits is actually quite limited. Two regions, which we designated A14 and A2, flank two of the putative metal-binding sites in the central part of the α subunit and are highly conserved between all of the α subunits that have been sequenced. Experiments performed using primers based on these regions allowed us to amplify a wide variety of α subunit cDNAs from different species, including one novel member of the family.

Previously reported sequence data about several integrin subunits suggested that integrin subunits are highly conserved between species. As expected, our consensus primers did work well with cDNA made from many different species. We obtained partial integrin cDNA sequences from a total of five species (human, guinea pig, mouse, pig, and rabbit). The α subunit deduced amino acid sequences were 86 to 97% identical between species. The cross-species identity for β subunits was 92 to 98%. Many of the cross-species differences are due to conservative substitutions. We did not observe a single case in which one or more amino acids were inserted or deleted in an integrin subunit in one species relative to another species. Because sequences are so highly conserved between species, we had no difficulty in determining whether a new cDNA sequence obtained from a given species encodes a truly novel integrin subunit or whether it merely encodes the homolog of a previously sequenced subunit from another species.

In this report, we present evidence for the existence of one

Figure 5. Partial deduced amino acid sequences of integrin β subunits. Sequences shown include previously reported β subunit sequences and β subunit sequences determined from the experiments presented here (see Table 2). Gaps (−) have been introduced in order to maximize the alignment. Amino acid residues conserved in all β subunits are indicated by asterisks; residues conserved in all but one subunit are indicated by dots. Residues in mouse, guinea pig, pig and rabbit sequences which differ from those in the human sequence are underlined. Abbreviations for the amino acids are given in the legend to Figure 1.



novel α subunit in airway epithelial cells and one novel β subunit in leukocytes. Comparison of previously reported integrin sequences with the novel cDNA sequences we identified strongly suggests that these novel cDNAs do in fact encode integrin subunits. Definitive proof of this will depend upon determination of the complete cDNA sequences and identification of the corresponding integrin subunit proteins. We have temporarily designated the novel α subunit sequence as α_A since it was detected in airway epithelial cells. The designation does not imply that expression of α_A is restricted to airway epithelial cells. This designation is rather arbitrary: some α subunits are designated by letter and some by number. All numbered α subunits associate with β_1; we prefer not to designate the novel α subunit with a number because we have no data about whether it associates with β_1. A more permanent nomenclature will have to await further characterization of α_A. The α_A sequence is one of six α subunit partial cDNA sequences identified in guinea pig airway epithelial cells. Each of the other five sequences is very similar (88 to 92% amino acid identity) to one of the α subunit sequences previously determined from another species, indicating that all five encode guinea pig homologs of previously sequenced α subunits from other species. The α_A partial sequence is only 24 to 60% identical to previously reported α subunits, indicating that α_A is a truly novel integrin α subunit cDNA. The α_A partial sequence is more closely related to the α_4 sequence, but it is not clear whether this implies any functional similarity between α_A and α_4. We identified novel cDNAs apparently encoding an integrin β subunit in human and mouse lymphoma cell lines, in a mouse macrophage-like cell line, and in a mixed population of rabbit leukocytes (mostly macrophages) obtained by BAL, but not in the epithelial or endothelial cells we studied. Since the previously sequenced β subunits are designated β_1 through β_6, we chose to designate the novel β subunit as β_7. The β_7 partial sequence is more similar to the β_2 sequence (61% identical) than to any of the other known β subunit sequences (40 to 55% identical). Additional studies using other methods will need to be performed in order to determine whether β_7 is restricted to leukocytes, as the β_2 subunit is thought to be. The relationship of the α_A and β_7 subunits to recently discovered integrin subunits such as the melanoma laminin receptor α subunit (14) and the lymphocyte β_p subunit (15, 16), for which no sequence data have been reported, cannot be determined from our data.

We were unable to identify some of the known α and β subunit cDNAs in any of the cells we studied. This may in part be explained by the relatively narrow cell type distribution of some integrin subunits, including α_{IIb} and β_4. The cells we studied may have very little or no mRNA encoding these integrin subunits. Further experience with the α and β subunit consensus primers in a wider variety of cell types should help to clarify this issue. In other cases, primers may be ill-suited for the amplification of certain known or novel integrin subunits. It is likely, for example, that the A2AR primer will not efficiently prime synthesis of the α_M or α_X cDNAs because of a marked primer-cDNA mismatch near the 3′ end of the primer (see Figure 1). We are not certain that this mismatch will prevent amplification since we have not yet studied cells expected to contain α_M or α_X with this primer. Another problem may arise when many different α or β subunits are expressed in the same cell: subunits that are less abundant or are amplified less efficiently may be difficult to identify. We used a technique we call "restriction-reamplification" to help overcome this problem (see RESULTS).

The partial cDNA clones generated with PCR are valuable reagents. These clones could be used directly to produce probes to be used for studying the distribution and regulation of integrin mRNAs by Northern hybridization or *in situ* hybridization. The probes can also be used to screen cDNA libraries in order to determine full-length sequences (21). Novel integrin subunit sequence information could then be used to produce synthetic peptides or fusion proteins to be used as immunogens. Antibodies produced with this approach would be used to identify and characterize the novel integrin subunit proteins.

Homology PCR has several advantages compared to other approaches for identifying and obtaining sequence information about novel integrin subunits. Affinity chromatography has been widely used as a method for identifying integrins, but some novel integrins may be difficult to identify with affinity chromatography because the identity of the ligand is unknown or because the ligand cannot be easily purified and immobilized in a functional state. In contrast, homology PCR requires no information about the ligand. The other major technique used for the identification of novel integrins has been the production of monoclonal antibodies to surface molecules on the cell of interest, followed by functional screening of hybridoma cells. This technique may not be applicable to some integrins due to poor immunogenicity or to the lack of a suitable *in vitro* assay for functional screening. While this approach has been extremely productive, it is considerably more laborious and time-consuming than homology PCR. The relationship of integrins identified with novel antibodies to previously identified integrins is not always clear. In contrast, since homology PCR provides partial sequence data, it should always be possible to unambiguously identify novel integrin subunits. Unlike the other two approaches used to identify integrins, homology PCR is well suited for the direct identification of integrins in BAL fluid cells and normal and diseased tissue as well as in cultured cells.

Acknowledgments: This work was supported in part by funds provided by the Cigarette and Tobacco Surtax Fund of the State of California through the Tobacco-Related Disease Research Program of the University of California, by a grant from the American Lung Association of California Research Program of the American Lung Association of California, and by Grants HL/AI-33259 and Pulmonary Vascular SCOR HL-19155 from the National Institutes of Health. Dr. Erle was supported by Training Grant HL-07155 from the National Institutes of Health and is an Edward Livingston Trudeau Scholar of the American Lung Association. Dr. Rüegg was supported by a grant from the Swiss National Science Foundation.

References

1. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* 238:491–497.
2. Ruoslahti, E., and F. G. Giancotti. 1989. Integrins and tumor cell dissemination. *Cancer Cells* 1:119–126.
3. De Strooper, B., B. Van der Schueren, M. Jaspers *et al.* 1989. Distribution of the beta 1 subgroup of the integrins in human cells and tissues. *J. Histochem. Cytochem.* 37:299–307.
4. Chen, W.-T., J.-M. Chen, and S. C. Mueller. 1986. Coupled expression and colocalization of 140K cell adhesion molecules, fibronectin, and laminin during morphogenesis and cytodifferentiation of chick lung cells. *J. Cell Biol.* 103:1073–1090.
5. Doerschuk, C. M., R. K. Winn, H. O. Coxson, and J. M. Harlan. 1990. CD18-dependent and -independent mechanisms of neutrophil emigration

in the pulmonary and systemic microcirculation of rabbits. *J. Immunol.* 144:2327–2333.
6. Rosen, H., and S. Gordon. 1990. The role of type 3 complement receptor in the induced recruitment of myelomonocytic cells to inflammatory sites in the mouse. *Am. J. Respir. Cell Mol. Biol.* 3:3–10.
7. Ismail, G., M. L. Morganroth, R. F. Todd, III, and L. A. Boxer. 1987. Prevention of pulmonary injury in isolated perfused rat lungs by activated human neutrophils preincubated with anti-Mol monoclonal antibody. *Blood* 69:1167–1174.
8. Horgan, M. J., S. D. Wright, and A. B. Malik. 1989. Protective effect of monoclonal antibody IB4 directed against leukocyte adhesion glycoprotein CD18 in reperfusion lung injury. *Am. Rev. Respir. Dis.* 139(Suppl.): A303. (Abstr.)
9. Kaslovsky, R. A., M. J. Horgan, J. Everitt, S. D. Wright, and A. B. Malik. 1989. Leukocyte CD18 adhesive glycoprotein is involved in neutrophil-dependent pulmonary edema induced by opsonized zymosan. *Am. Rev. Respir. Dis.* 139(Suppl.):A303. (Abstr.)
10. Humphries, M. J., K. Olden, and K. M. Yamada. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science* 233:467–470.
11. Tamkun, J. W., D. W. DeSimone, D. Fonda *et al.* 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* 47:271–282.
12. Bogaert, T., N. Brown, and M. Wilcox. 1987. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell* 51:929–940.
13. MacKrell, A. J., B. Blumberg, S. R. Haynes, and J. H. Fessler. 1988. The lethal myospheroid gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin β subunits. *Proc. Natl. Acad. Sci. USA* 85:2633–2637.
14. Kramer, R. H., K. A. McDonald, and M. P. Vu. 1989. Human melanoma cells express a novel integrin receptor for laminin. *J. Biol. Chem.* 264:15642–15649.
15. Holzmann, B., B. W. McIntyre, and I. L. Weissman. 1989. Identification of a murine Peyer's patch–specific lymphocyte homing receptor as an integrin molecule with an α chain homologous to human VLA-4α. *Cell* 56:37–46.
16. Holzmann, B., and I. L. Weissman. 1989. Peyer's patch–specific lymphocyte homing receptors consist of a VLA-4–like α chain associated with either of two integrin β chains, one of which is novel. *EMBO J.* 8:1735–1741.
17. Gresham, H. D., J. L. Goodwin, P. M. Allen, D. C. Anderson, and E. J. Brown. 1989. A novel member of the integrin receptor family mediates Arg-Gly-Asp–stimulated neutrophil phagocytosis. *J. Cell Biol.* 108: 1935–1943.
18. Libert, F., M. Parmentier, A. Lefort *et al.* 1989. Selective amplification and cloning of four new members of the G protein–coupled receptor family. *Science* 244:569–572.
19. Gould, S. J., S. Subramani, and I. E. Scheffler. 1989. Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. *Proc. Natl. Acad. Sci. USA* 86: 1934–1938.
20. Kamb, A., M. Weir, B. Rudy, H. Varmus, and C. Kenyon. 1989. Identification of genes from pattern formation, tyrosine kinase, and potassium channel families by DNA amplification. *Proc. Natl. Acad. Sci. USA* 86: 4372–4376.
21. Sheppard, D., C. Rozzo, L. Starr, V. Quaranta, D. J. Erle, and R. Pytela. 1990. Complete amino acid sequence of a novel integrin β subunit (β_6) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.* 265:11502–11507.
22. Adler, K. B., J. E. Schwarz, M. J. Whitcutt, and R. Wu. 1987. A new chamber system for maintaining differentiated guinea pig respiratory epithelial cells between air and liquid phases. *BioTechniques* 5:462–465.
23. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387–395.
24. D'Souza, S. E., M. H. Ginsberg, T. A. Burke, S. C. Lam, and E. F. Plow. 1988. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* 242:91–93.
25. Pytela, R. 1988. Amino acid sequence of the murine Mac-1 alpha chain reveals homology with the integrin family and an additional domain related to von Willebrand factor. *EMBO J.* 7:1371–1378.
26. Larson, R. S., A. L. Corbi, L. Berman, and T. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1 alpha subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* 108:703–712.
27. Ignatius, M. J., T. H. Large, M. Houde *et al.* 1990. Molecular cloning of the rat integrin α_1-subunit: a receptor for laminin and collagen. *J. Cell Biol.* 111:709–720.
28. Takada, Y., and M. E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109:397–407.
29. Tsuji, T., F. Yamamoto, Y. Miura *et al.* 1990. Characterization through cDNA cloning of galactoprotein b3 (Gap b3), a cell surface membrane glycoprotein showing enhanced expression on oncogenic transformation: identification of Gap b3 as a member of the integrin superfamily. *J. Biol. Chem.* 265:7016–7021.
30. Tamura, R. N., C. Rozzo, L. Starr *et al.* 1990. Epithelial integrin $\alpha_6\beta_4$: complete primary structure of α_6 and variant forms of β_4. *J. Cell Biol.* 111:1593–1604.
31. Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the alpha 4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO J.* 8:1361–1368.
32. Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.
33. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor α subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.
34. Poncz, M., R. Eisman, R. Heidenreich *et al.* 1987. Structure of the platelet membrane glycoprotein IIb. Homology to the α subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.
35. Arnaout, M. A., S. K. Gupta, M. W. Pierce, and D. G. Tenen. 1988. Amino acid sequence of the alpha subunit of human leukocyte adhesion receptor Mol (complement receptor type 3). *J. Cell Biol.* 106:2153–2158.
36. Corbi, A. L., T. K. Kishimoto, L. J. Miller, and T. A. Springer. 1988. The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit. Cloning, primary structure, and relation to the integrins, von Willebrand factor and factor B. *J. Biol. Chem.* 263: 12403–12411.
37. Corbi, A. L., L. J. Miller, K. O'Connor, R. S. Larson, and T. A. Springer. 1987. cDNA cloning and complete primary structure of the alpha subunit of a leukocyte adhesion glycoprotein, p150,95. *EMBO J.* 6:4023–4028.
38. Tominaga, S. 1988. Murine mRNA for the β-subunit of integrin is increased in BALB/c-3T3 cells entering the G1 phase from the G0 state. *FEBS Lett.* 238:315–319.
39. Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell* 48:681–690.
40. Law, S. K., J. Gagnon, J. E. Hildreth, C. E. Wells, A. C. Willis, and A. J. Wong. 1987. The primary structure of the beta-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor. *EMBO J.* 6:915–919.
41. Fitzgerald, L. A., B. Steiner, S. C. J. Rall, S. S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin." *J. Biol. Chem.* 262:3936–3939.
42. Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.* 9:757–763.
43. Hogervorst, F., I. Kuikman, A. E. G. K. von dem Borne, and A. Sonnenberg. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J.* 9:765–770.
44. Ramaswamy, H., and M. E. Hemler. 1990. Cloning, primary structure and properties of a novel human integrin β subunit. *EMBO J.* 9:1561–1568.
45. Suzuki, S., Z.-S. Huang, and H. Tanihara. 1990. Cloning of an integrin β subunit exhibiting high homology with integrin β_3 subunit. *Proc. Natl. Acad. Sci. USA* 87:5354–5358.

Eur. J. Biochem. 199, 425–433 (1991)

00142956910045 9W

Molecular cloning of the human α6 integrin subunit

Alternative splicing of α6 mRNA and chromosomal localization of the α6 and β4 genes

Frans HOGERVORST[1], Ingrid KUIKMAN[1], Ad Geurts VAN KESSEL[2] and Arnoud SONNENBERG[1]

[1] Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, The Netherlands
[2] Department of Human Genetics, University of Nijmegen, The Netherlands

(Received January 23/April 8, 1991) – EJB 91 0131

We have isolated cDNAs encoding the α6 subunit from a λ gt11 expression library from human keratinocytes by combined screening with a rabbit polyclonal anti-α6 antibody and the polymerase chain reaction. The α6 subunit encoded by this cDNA consists of 1050 amino acids with a 991-amino-acid extracellular, a 23-amino-acid transmembrane and a 36-amino-acid cytoplasmic domain. The extracellular domain contains three putative divalent cation-binding sites and nine potential N-linked glycosylation sites. From a cDNA library from normal human mammary gland cells two different cDNAs for α6 were isolated, one of which is identical to the above cDNA. The two α6 subunits, called α6A and α6B, encoded by the two cDNAs each have a unique cytoplasmic domain, that of α6B being 18 amino acids longer than that of α6A.

Different carcinoma cell lines contain transcripts for both α6 subunits. K562 leukemic cells have little α6A or α6B mRNAs. The overall level of expression varies in the carcinoma cell lines, but reflects α6 cell surface expression. In A375 melanoma cells, however, cell surface expression of α6 was low in spite of a high level of mRNA. This suggests that other mechanisms may be involved in regulating the expression of α6 on the surface of these cells. The mRNA for both α6 subunits is around 6 kb.

The α6 subunits are similar to other α subunits (26–31% identity with cleaved α subunits) of the integrin family but they are more similar to the α3 subunit (40% identity). This high degree of similarity may be the basis for their functional resemblance since both α3 and α6 subunits, when associated with β1, function as laminin receptors and bind to the long arm of laminin. The genes for α6 and β4, the alternative β subunit with which α6 combines on certain epithelial cells, were mapped to chromosome 2 and 17q11-qter, respectively.

The α6β1 and α6β4 complexes are two members of the large family of integrins involved in cell – cell and cell – matrix interactions [1, 2]. In addition, the α6β1 is part of the very late antigen (VLA) subfamily of integrins, the members of which have unique α subunits but share a common β1 subunit [3]. The α6β1 integrin is expressed on a variety of cell types [4, 5] and is involved in adhesion to laminin [6]. It specifically interacts with a site on the long arm of laminin in the elastase-derived fragment E8 [7, 8]. Another member of the VLA protein family, VLA-3, also functions as a laminin receptor [9] and binds to a similar region on laminin as VLA-6 [10]. However, VLA-3 differs from VLA-6 in that it can also bind to fibronectin and collagen [11], whereas VLA-6 appears to be specific for laminin. In addition to binding to laminin, the VLA-3 and VLA-6 integrins share many structural features. Both the α3 and α6 subunits associate with the same β1 subunit, have similar molecular masses and consist of disulfide-linked heavy and light chains which both appear to be glycosylated [4, 12, 13]. Two other members of the VLA protein family, VLA-1 [14] and VLA-2 [15, 16], which can also bind to laminin, are structurally only distantly related to the VLA-3 and VLA-6 integrins; i.e. the α subunits of VLA-1 and VLA-2 are single polypeptides whereas those of VLA-3 and VLA-6 are cleaved polypeptides. Moreover, VLA-1 has been shown to act as a receptor for the laminin fragment E1, containing the core and short arms and proximal portions of the short arms of laminin [17, 18].

The function of α6β4 is not yet clear. In one study using colon carcinoma cells it was suggested to be a laminin receptor [19], but this could not be confirmed in a study using mammary tumor cells [8]. The α6β4 integrin has been localized on the basal surfaces of polarized epithelial cells [20]. This result, combined with the recent observation that α6β4 is localized in hemidesmosomes of epidermal cells [21, 22], strongly suggests that it has an important function in the adhesion of cells to the extracellular matrix.

Many α and β subunits [23–47] have now been sequenced and significant similarities have been found among the different α subunits as well as the β subunits. Both α and β subunits are transmembrane glycoproteins with relatively short cyto-

Correspondence to A. Sonnenberg, Department of Immunohematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, NL-1066 CX Amsterdam, The Netherlands

Abbreviations. VLA, very late antigen; PCR, polymerase chain reaction; [^{35}S]dATP[αS] 2′-deoxyadenosine 5′-α-[^{35}S]thiotriphosphate.

Note. The novel nucleotide sequence data published here have been deposited with the EMBL sequence data bank and are available under the accession number X59512. The novel amino acid sequence data have been deposited with the EMBL sequence data bank.

plasmic domains. An exception is the β4 subunit which has an unusually large cytoplasmic domain of around 1000 amino acids [42, 45].

In this paper we describe the cloning and sequence analysis of the human α6 subunit from keratinocytes and mammary cells. In addition, we report on alternative splicing of the α6 mRNA and present the chromosomal localization of the α6 and β4 genes. After the investigations reported in this paper were completed, a publication appeared in which Tamura et al. [47] describe the cloning of a cDNA for the α6 subunit from pancreatic carcinoma cells. Their results and ours will be compared.

MATERIALS AND METHODS

cDNA cloning

A phage λ gt11 cDNA library of human keratinocytes (Clontech Laboratories, Inc., Palo Alto, CA) comprising 500000 recombinant plaques was screened with a rabbit polyclonal α6 antiserum [20] using the protoblot immunoscreening system of Promega. Sixty positive plaques were selected and subjected to an additional screening by polymerase chain reaction (PCR) using synthetic oligonucleotides. Two unique λ gt11 primers complementary to a portion of the β-galactosidase gene on the right (5′-GGTGGCGACGACTCCTGGAGACCCG-3′) and left arms (5′-TTGACACCAGACCAACTGGTAATG-3′) of λ DNA and two degenerate primers, a sense and antisense primer (128-fold degenerate) corresponding to the conserved amino acid sequence WKXGFFK in many α subunits, were used. The X was assumed to be a C because of the expected amino acid sequence similarity between the α3 [46, 48] and α6 subunits. Each primer contained an additional eight nucleotides encoding a restriction enzyme site (*Eco*RI or *Hin*dIII) for subcloning of the fragments in pUC18 plasmids. Positive clones were plaque-purified and the λ cDNAs inserts subcloned into pUC18 vectors for restriction analysis. A cDNA library made in λ gt11 from normal human mammary cells (Clontech Laboratories, Inc., Palo Alto, CA) was screened with ^{32}P-labeled insert probes from α6 cDNA clones.

cDNA synthesis and polymerase chain reaction

RNA (5 μg) from cell lines was isolated by the ureum/lithium chloride method [49]. First strand cDNA was prepared with a Promega cDNA kit, using 5 μg total cellular RNA/25 μl. Distilled water was then added to 50 μl and 2.5 μl of the cDNA reaction was used in a 50-μl PCR containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 9.0, 0.1% gelatin, 0.1% Triton X-100, 0.2 mM dNTPs, 160 ng primers and 2 U *Taq* polymerase. After 5 min at 94°C, 35 cycles of amplification were performed in a Perkin Elmer Cetus thermocycler using the following conditions: denaturation for 1 min at 94°C, annealing for 1.5 min at 60°C and extension for 1.5 min at 72°C. These 35 cycles were followed by a final extension for 5 min at 72°C and shutdown at 4°C. The primers used for the PCR were: (a) sense primer, positions 2656–2675 (5′-CTAACGGAGTCTCACAACTC-3′) and (b) antisense primer, positions 3480–3499 (5′-ACTCTGAAATCAGTCCTCAG-3′). Amplified DNA was analyzed on 1.25% agarose gels.

Sequence analysis and similarities

DNA sequencing was performed by the dideoxy-chain-termination method [50] using a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) and 2′-deoxyadenosine 5′-α-[^{35}S]thiotriphosphate ([^{35}S]dATP[αS]) as radioactive nucleotide. Sequences were obtained from one end of subcloned restriction fragments by using universal M13/pUC sequencing primers. The internal sequence of large fragments were obtained from oligonucleotide primers that were synthesized on the basis of the preceding sequence. Sequence artefacts were avoided by determining all sequences using both dGTP and the nucleotide deoxyinosine triphosphate (dITP) and by sequencing both strands of the cDNA insert. Sequence data were analyzed using GCG software [51].

Northern blot analysis

Total cellular RNA isolated from cell lines which express high levels (HBL-100 and OVCAR-4), low levels (T47D and A375) and no (K562) α6 protein was electrophoresed in a 1% agarose gel containing formaldehyde and transferred to nitrocellulose by a standard procedure [52]. Hybridizations were carried out for 16 h at 64°C with ^{32}P-labelled insert probes in 3 × NaCl/Cit (1 × NaCl/Cit = 0.15 M sodium chloride and 15 mM sodium citrate, pH 7.0) containing 10% dextran sulphate, 5 × Denhardt's solution (Denhardt's solution = 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% bovine serum albumin) and 50 μg/ml salmon sperm DNA. The filters were washed once with 3 × NaCl/Cit, 0.1% SDS for 30 min at 64°C and twice with 2 × NaCl/Cit, 0.1% SDS for 30 min at 64°C, dried and exposed to Kodak XAR-5 film with Dupont Cronex Lightning-Plus intensifying screens. The following probes were used in this study: (a) a 4.3-kb cDNA fragment of K134, a cDNA clone from human β4 [42]; (b) a 2.3-kb cDNA fragment coding for the extracellular part of the human β1 subunit, isolated in our laboratory from the same human keratinocyte library from which we cloned the α6 and β4 subunits; (c) a 600-bp fragment from clone A33, a cDNA clone from human α6 (this report).

Chromosomal localization of α6 and β4 genes

For our chromosomal localization studies a panel of well defined human-rodent somatic cell hybrids was used [53]. These hybrid lines were isolated after fusion of human cells, obtained from different donors, with rodent cell lines deficient in hypoxanthine phosphoribosyltransferase or thymidine kinase. Parental and hybrid cells were grown in HAMF10 or RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. For the chromosomal analysis of the hybrid cell lines, air-dried chromosome spreads were made according to standard procedures [54]. Of each cell line at least 16 metaphases were examined using R-banding after heat denaturation. The cells used for chromosome analysis and DNA extraction were always derived from the same culture batch.

RESULTS AND DISCUSSION

Isolation of cDNA clones for integrin α6 subunit

After immunological screening and PCR analysis of a human keratinocyte library (see Materials and Methods), 12 reactive clones were obtained. The longest cDNA clone which

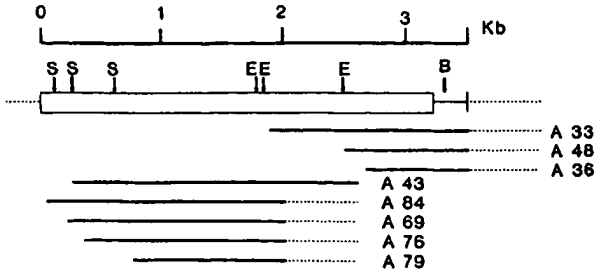


Fig. 1. *Restriction map of α6 cDNA clones.* The open reading frame is shown as an open box. The solid lines indicate the size and order of the various cDNA clones. The 3′ ends of all clones, except clone A43 have not been characterized or sequenced and are indicated by a dotted line. Relevant restriction sites are *Eco*RI (E), *Sma*I (S) and *Bam*HI (B)

is a representative of five different clones with similar insert sizes, and two smaller clones were plaque-purified and their cDNA inserts were subcloned into pUC18 (Fig. 1). Sequencing of the three cDNA clones revealed that they were colinear and only differed in size. Furthermore, the sequence indicated typical features of α subunits: a transmembrane domain of 23 amino acids, followed by a short cytoplasmic domain of 36 amino acids including the GFFKR sequence common to all integrin α subunits. When the 60 clones were rescreened with a radiolabeled probe from the 5′ portion of λ A33, a 600-bp *Eco*RI fragment, an overlapping cDNA clone (λ A43) was isolated that extended further in the 5′ direction. This clone lacked the 3′ portion containing the WKXGFFK sequence and consequently could not give a reaction product in the PCR. More overlapping cDNA clones were obtained by screening the keratinocyte λ gt11 cDNA library with the radiolabeled probes from the 5′ portion of λ A33. One (λ A84) of the newly isolated cDNA clones contained the N-terminus of α6 [54, 55], but lacked part of the signal peptide. An additional round of screening, using a 5′ fragment of λ A84, a 450-bp *Sma*I fragment, did not result in cDNA clones covering the entire signal peptide of α6.

When a cDNA library from normal human mammary gland cells in λ gt11 was screened using an α6 probe, again no clones extending further in the 5′ direction were obtained. However, we did obtain clones (Fig. 3; Fig. 4, lanes 8–12) which code for two different α6 subunits: α6A and α6B. Partial sequences of the α6A clone revealed that this clone is identical to the α6 cDNA identified in keratinocytes. The α6B clone is a variant form of α6A with a unique cytoplasmic tail as described in detail below.

Nucleotide and deduced amino acid sequence

The nucleotide sequence and the deduced amino acid sequence of α6A are shown in Fig. 2. The mature α6A subunit contains a large extracellular domain of 991 amino acids, a single transmembrane domain of 23 amino acids, and a short cytoplasmic domain of 36 amino acids. Its predicted molecular mass is 117111 Da. There are nine potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is not Pro) in the extracellular domain of α6. If all of these sites are occupied with carbohydrate chains (average molecular mass of 2500 Da), the total molecular mass would be 139.5 kDa. This value is consistent with that estimated from the mobility of the α6 subunit on SDS/PAGE of 140 kDa [4, 20].

The N-terminal portion of the α6A sequence is composed of seven similar repeating domains (domain I, residues 19–69; II, 72–129; III, 160–212; IV, 233–286; V 291–346; VI, 352–404; VII, 407–463). Putative divalent cation binding sites are present in domains V, VI and VII. These sites have been described for all other integrin α subunits and are similar to the EF-hand consensus metal binding sites of a number of Ca^{2+} and Mg^{2+} binding proteins, i.e. troponin [56]. Only the α subunits of the fibronectin and vitronectin receptors, and the α subunit of the platelet glycoprotein IIb-IIIa complex contain a fourth potential divalent cation binding site in domain IV. The presence of divalent cation binding sites is consistent with the dependence of the function of integrins on divalent cations.

In the α6B sequence 130 nucleotides in the coding region for the cytoplasmic domain of α6A are deleted (Fig. 2). This results in a frameshift, generating a cytoplasmic domain of 54 novel amino acids (Fig. 3), which produces an α6B subunit that is 2.6 kDa larger than α6A. A distinctive feature of the cytoplasmic domain of α6B is the high number of charged amino acids (24 out of 54). It is also of interest that the highly conserved cytoplasmic tail sequence GFFKR in all α subunits is still present in α6B.

We suggest that the two cytoplasmic domains of α6A and α6B are contained within two separate exons and that the two cDNA variants are generated by alternative splicing of mRNA. This view is compatible with the present knowledge of the genomic organization of the three integrin α subunits studied so far, which all have an intron between the transmembrane domain and the cytoplasmic tail [57–59]. Furthermore, alternative splicing of integrin α and β subunit transcripts appears to be quite common. This phenomenon has been reported for the mRNAs of at least five different integrin subunits [47, 57, 60–62].

Structural features of α6A and α6B

Biosynthetic studies have indicated that the α6 subunit is synthesized as a single precursor which is proteolytically processed into heavy and light chains which are linked by disulfide bonding [13]. Because light chains of two different sizes (31 and 30 kDa) have been found in α6 preparations of platelets and of different cell lines, which apparently were not due to differences in glycosylation, it was suggested that cleavage of the precursor occurs at two alternative sites [20, 63]. The α6 sequence contains four dibasic sequences in a region in which cleavage may occur to yield light chains of the expected sizes. Two dibasic residues (positions 877 and 879) immediately follow each other and two others are located downstream, towards the carboxy terminus, at a distance of 12 (position 891) and 20 residues (position 899). A double dibasic residue in the same position as that in α6 has been found in the α3 subunit [46]. Furthermore, dibasic residues, separated by a single amino acid, were found in a similar position in αIIb [31] and PS2α [24], and a single dibasic cleavage site was found in α5 [23] and αv [32]. This suggests that there is a primary cleavage site at this position. In addition, the α3 and PS2α subunits have a second dibasic cleavage site possibly corresponding to the dibasic site 899 in α6. A single residue in this position is seen in the α subunit of GPIIb-IIIa and found to be responsible for cleavage of a minor proportion of GPIIb molecules [64]. The third dibasic site at position 891 is apparently unique to α6 and it seems possible that alternative use of this third site and the dibasic cleavage at position 899 yields the two light chains of different sizes.

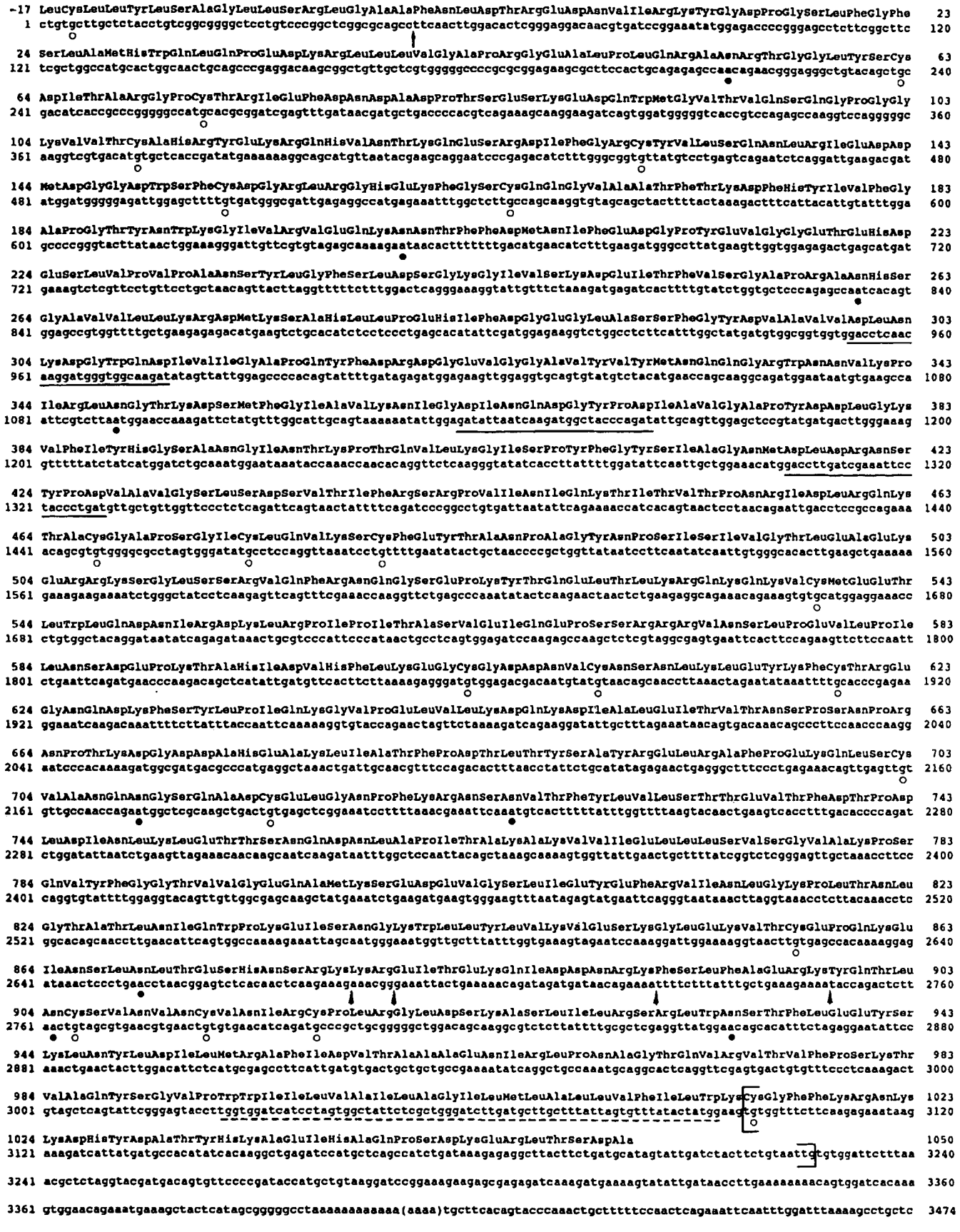


Fig. 2. *The cDNA sequence and deduced amino acid sequence of human α6A subunit.* The beginning of the mature protein is indicated by an arrow. The cysteine residues are marked by open circles and the potential N-linked glycosylation sites by closed circles. The position of the transmembrane region (dashed line) and the putative divalent cation-binding sites (solid line) are indicated. Arrowheads denote the position of the possible dibasic cleavage sites of heavy and light chains of α6. The deletion of 130 nucleotides found in cDNAs coding for α6B is indicated between brackets

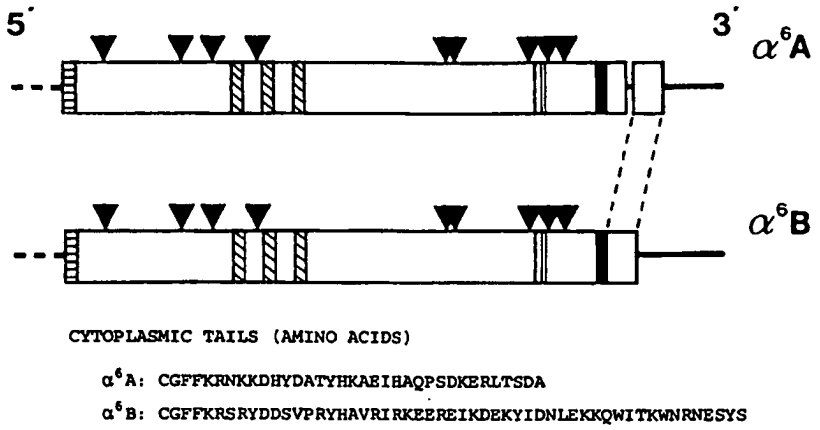


Fig. 3. *Schematic representation of α6A and α6B cDNAs.* The open reading frames (large and small boxes) are shown with the following regions indicated: the signal peptide (box with horizontal lines); three putative divalent cation binding domains (boxes with obliqued lines); potential dibasic cleavage sites (vertical lines) and the transmembrane region (darkened box). Thin black lines indicate untranslated sequences. The 5′ ends of the two α6 cDNAs including signal peptide have not been cloned and are indicated by a dotted line. The nine potential glycosylation sites are indicated by triangles. The deduced amino acid sequence (single-letter code) of the cytoplasmic regions are shown at the bottom of the figure

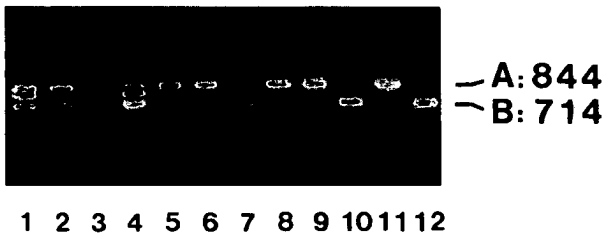


Fig. 4. *Detection by PCR amplification of mRNA transcripts coding for the α6A and α6B variants.* PCR was performed on cDNA synthesized A375 (lane 1), HBL100 (lane 2), K562 (lane 3), OVCAR-4 (lane 4) and T47D (lane 5) cell lines, on total phage DNA from keratinocyte (lane 6) and mammary cell (lane 7) libraries and on phage DNA from five clones that were selected from the mammary cell library by screening with a radiolabeled α6 cDNA probe (lanes 8–12). Primers 1 and 2 (see Materials and Methods) were used in all PCR reactions. The PCR products for α6A and α6B from HBL-100 cDNA were subcloned and sequenced. The two sequences appeared to be identical to those from the α6A and α6B cDNA clones isolated from the mammary cell library in λ gt11. The PCR products were separated on 1.25% agarose gels and stained with ethidium bromide

However, the two different α6 subunit cDNAs offer an alternative explanation for the two α6 light chains isolated from platelets and different cell lines [20]. PCR analysis showed that in A375, HBL-100, T47D and OVCAR-4 cells, cDNAs for both α6A and α6B are indeed present (Fig. 4). In all cell lines, a third PCR product migrating slightly faster than the A variant is also observed. A cDNA clone corresponding to this PCR product has as yet not been found in either the keratinocyte or the normal mammary cell libraries. Therefore, it still remains to be established whether this PCR product corresponds to yet another α6 splice variant or whether it is an artifact of the PCR technique. The possibility that contaminating genomic DNA was the source for the third PCR product, however, can be ruled out because (a) the PCR product remained detectable when poly(A)-rich RNA was used for synthesis of cDNA and (b) no PCR product was obtained when the reverse transcriptase reaction was omitted.

The α6 light chain encoded by the two cDNAs (α6A and α6B) contains three cysteines in identical positions as those in other α subunits which are thought to be involved in the covalent binding of the light chain to the heavy chain. Furthermore, the sequence of the light chain of both α6 subunits contains two possible N-linked glycosylation sites, which is in agreement with previous studies on the glycosylation of the α6 light chain [4, 20, 63].

Shaw et al. [65] reported that in macrophages treated with 4β-phorbol 12-myristate 13-acetate the cytoplasmic domain of the α6 subunit is phosphorylated, possibly at a serine or threonine residue. Although it is not known which α6 variant is expressed by macrophages, both α6 subunits have several serine and threonine residues in their cytoplasmic domains which may be targets for phosphorylation. It may be noteworthy that the α3 subunit is phosphorylated on serine residues [12] and that serine 1041 in α6A and its surrounding sequences are conserved in α3 and α6A. In addition, the cytoplasmic domains of α6A and α6B contain several tyrosine residues that could serve as potential phosphorylation sites.

Comparison with other α subunits

Comparison of the sequences of the two α6 subunits with those of other integrin α subunits shows that they are most similar to hamster α3 (40% identical). This similarity is most evident in the repeating domains V (54.5%), VI (58.5%) and VII (55.4% identical). For α6A, there is also considerable similarity with α3 in the cytoplasmic domain; 20 out of 36 residues are shared. This finding is remarkable because in general there is little similarity between the cytoplasmic domains of different α subunits. It may indicate functional similarity. Overall identities between α6 and other cleaved subunits are 31% for α5, 29% for αv and 26% for αIIB. Of the 20 cysteines in α6, 18 can be aligned with cysteines in the four cleaved α subunits.

Because both α3 and α6 integrins bind to laminin and may even recognize the same site on laminin, located in the E8 fragment on the long arm of laminin [7, 8, 10], we searched for sequences that are shared by these two subunits and, for example, were not present in α5. The α5 of sequence was chosen for comparison because the α5 integrin has a clearly different ligand specificity from that of the α6 integrin but, like α6, associates with β1 and is composed of two polypeptides. In

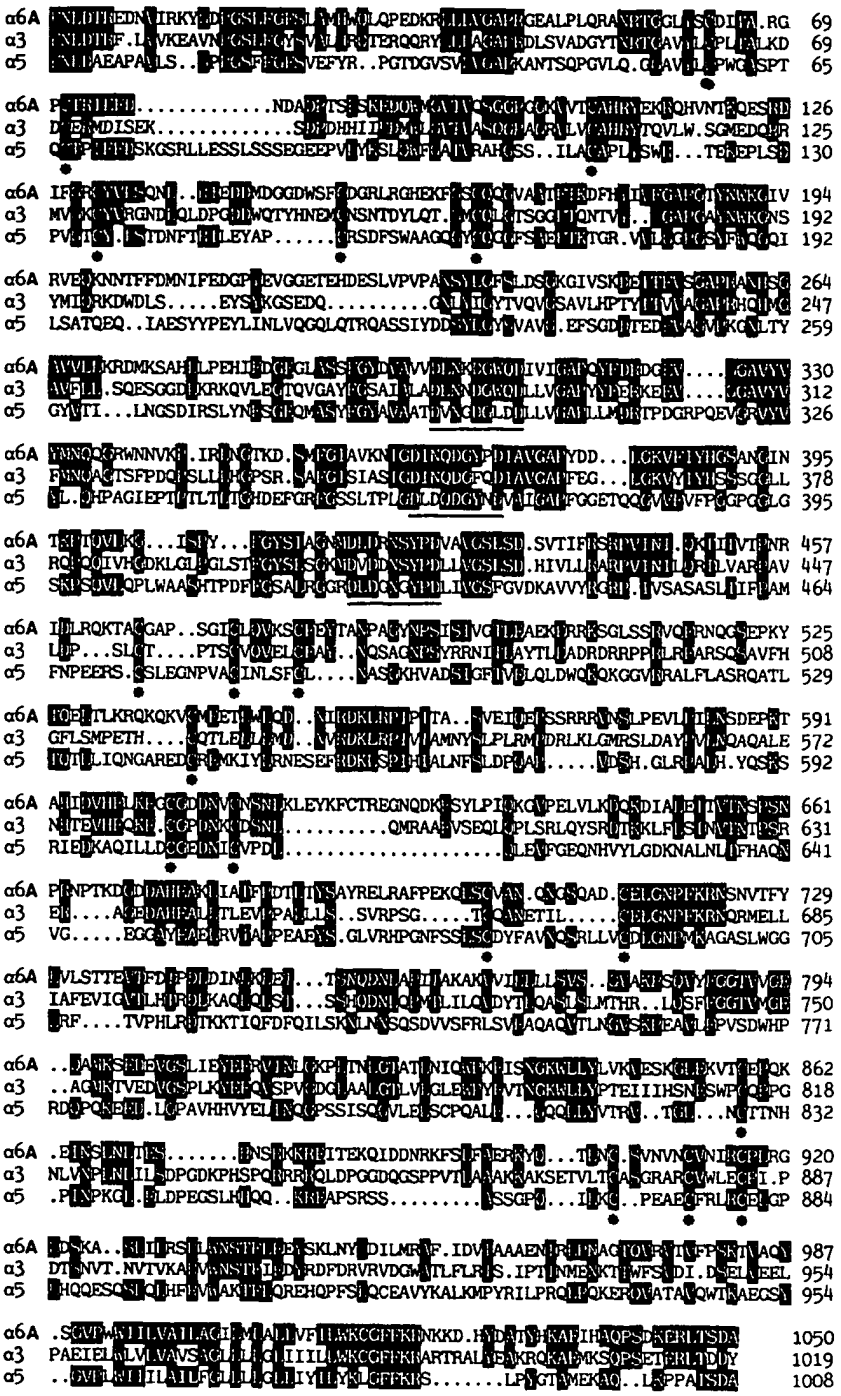


Fig. 5. *Alignment of the α6A protein sequence with other integrin α subunits.* The α6A sequence is compared to hamster α3 [46] and human α5 integrin subunit sequences [23, 27]. Amino acids identical between the α6A subunit and either of the two α subunit sequences are indicated by inverse printing. The three divalent cation binding sites are underlined. The cysteines residues conserved among the three α subunits sequences are marked by an asterisk (*)

the aligned sequences (Fig. 5), 211 amino acids were found to be exclusively shared by the α3 and α6A subunits. A region located between two cysteines (residues 714–858) contained 44 of these exclusively shared amino acids. The number of exclusively shared residues between α6 and α3 is considerably higher than that between α6 and α5: 17 residues exclusively shared. Furthermore, the exclusively shared amino acids between α6 and α5 appear not to be specifically concentrated in the region between cysteines 714–858, as is the case for α3 and α6. A possible involvement of this region in ligand binding, however, should be investigated in further studies using site-directed mutagenesis.

The deduced amino acid sequence of α6 from pancreatic carcinoma cells reported by Tamura et al. [47] is similar to that of α6A except at three positions (55, 300 and 743). At one of these positions (amino acid 55), we established the presence of a potential glycosylation site, which is also conserved in the sequence of the α3 subunit. This is also true for the amino

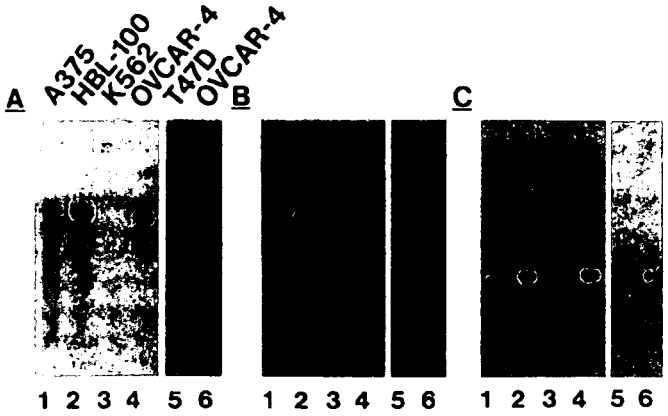


Fig. 6. *Northern blot analysis.* Total RNA (10 μg/lane) from A375, HBL-100, K562, OVCAR-4 and T47D cell lines was electrophoretically separated on 1% agarose/formaldehyde gels, transferred to nitrocellulose filters and probed with ^{32}P-labeled cDNAs encoding the α6 (A), β4 (B), and β1 subunits (C)

Table 1. *Correlation between the presence or absence of human chromosome and the β4 gene in 21 human-rodent somatic cell hybrids*

Chromosome	Chromosome/β4		No. of clones		Discordancy
	+/+	+/−	−/+	−/−	
					%
1	9	1	8	3	43
2	4	1	13	3	67
3	10	1	7	3	38
4	11	3	6	1	43
5	10	2	7	2	43
6	9	1	8	3	43
7	9	2	8	2	48
8	11	3	6	1	43
9	7	1	10	3	52
10	9	2	8	2	48
11	8	2	9	2	52
12	9	1	8	3	43
13	7	2	10	2	57
14	8	3	9	1	57
15	8	3	9	1	57
16	12	2	5	2	33
17[a]	16	0	0	4	0
18	9	2	8	2	48
19	9	2	8	2	48
20	12	3	5	1	38
21	11	2	6	2	38
22	10	1	7	3	38
X	11	4	6	0	48

[a] One hybrid containing an iso-17q chromosome was not included.

Table 2. *Correlation between the presence or absence of human chromosome and the α6 gene in 34 human-rodent somatic cell hybrids*

Chromosome	Chromosome/β4		No. of clones		Discordancy
	+/+	+/−	−/+	−/−	
					%
1	7	7	0	20	21
2	6	0	1	27	3
3	4	9	3	18	35
4	5	9	2	18	32
5	7	7	0	20	21
6	3	8	4	19	35
7	6	6	1	21	21
8	5	12	2	15	41
9	2	11	5	16	47
10	3	8	4	19	35
11	3	13	4	14	50
12	4	7	3	20	29
13	5	6	2	21	24
14	4	11	3	16	41
15	4	10	3	17	38
16	5	13	2	14	44
17	5	16	2	11	53
18	6	7	1	20	24
19	3	13	4	14	50
20	6	14	1	13	44
21	5	14	2	13	47
22	4	14	3	13	50
X	4	14	3	13	50

acid found by us at position 743. The basis for these different findings is not clear, but one possibility is that they reflect polymorphisms of the α6 gene.

Northern blot analysis

Northern blot analysis, performed with an α6 probe which detects both α6A and α6B of cells which contain cDNAs for these two different subunits (see Fig. 3), revealed a single mRNA of 6 kb. Apparently, in our Northern blots the different sizes of α6A and α6B (i.e. 130 nucleotides) is not detectable. The mRNA is strongly expressed by HBL-100, OVCAR-4 and A375 cells and weakly by T47D cells. In K562 leukemic cells, expression of α6 mRNA could not be detected, although the PCR showed the presence of a few α6A and α6B transcripts. Hybridization of the same blot with a β1 cDNA probe showed strong expression of this subunit in all five cell lines. In contrast, the levels of β4 mRNA were high in HBL-100 and T47D cells, extremely low in OVCAR-4 and A375, while no β4 mRNA was detectable in K562 cells. Control hybridization with glyceraldehyde-phosphate dehydrogenase showed no variation in expression (not shown). With the exception of A375 cells, on whose surface α6 is weakly expressed but whose α6 mRNA levels are high, the RNA expression data are consistent with the known cell-surface expression of α6β1 and α6β4 complexes on these cells [8, 20]. Although the mechanism responsible for the difference between cell surface and α6 mRNA expression in A375 cells is not known, it indicates that, in addition to the level of mRNA, other mechanisms can be involved in regulating the expression of α6 at the cell surface.

Chromosomal localization of α6 and β4

To determine the chromosomal localization of the α6 and β4 genes, the respective cDNA inserts were hybridized to panels of human−mouse or human−hamster cell lines containing various subsets of human chromosomes (Tables 1 and 2). The score of 0% discordancy (β4; Table 1) indicates that positive hybridizing fragments were detected in all cell lines retaining human chromosome 17 and not in any of the lines lacking this chromosome. For all other chromosomes a dis-

cordancy score of at least 33% was observed. From this result we conclude that the β4 gene must be located on human chromosome 17. One cell line had retained only part of chromosome 17 (in the form of an isochromosome 17q) and showed a positive signal with the β4 probe used. This indicates that the β4 gene is present on 17q. In order to refine its localization further, two hybrid cell lines, obtained after fusion of t(15,17) (q22;q11) positive acute promyelocytic leukemia cells with a murine cell line containing either of the reciprocal translocation products in the absence of a normal 17, were used [66]. Hybrid C73/PJT2A1, containing the $15q^+$ derivative of the translocation was positive, whereas hybrid P12.3B6, containing the 17q derivative was negative. From this we conclude that the β4 gene maps to the q11-qter region of human chromosome 17. A discordancy score of 3% was obtained for the α6 gene and chromosome 2 (Table 2). Again, for all other human chromosomes discordancy scores were invariably higher, which indicates that the α6 gene resides on chromosome 2. One discordant hybrid clone showed a positive signal with the α6 probe but was cytogenetically scored negative for chromosome 2. Possibly, a rearrangement of this chromosome has occurred during or after cell fusion, which has hampered its identification. This phenomenon is frequently observed in somatic cell hybrids. The genes for α6 and β4 are physically close to the genes of three other integrin subunits, the α and β subunits of the GPIIb-IIIa complex, which have been mapped to 17q21-23, and the vitronectin receptor α subunit which has been assigned to chromosome 2 [67]. The α3 subunit is also located on chromosome 17, but has not been localized further [68]. Thus, genes encoding various integrin subunits appear to be located on human chromosomes 2 and 17, respectively.

The authors thank Dr D. Sheer and Dr. D. Ledbetter for providing t(15;17)-derived hybrid cell DNAs, Mrs E. van Drunen and Mrs L. Admiraal for technical assistance and Mrs M. van de Ende for synthesizing the oligonucleotides. This work was supported by a grant from the Foundation for Medical Research MEDIGON, which is subsidized by the Netherlands Organization for Scientific Research (NWO) (grant 900-526-106) and by a grant from the Dutch Cancer Society (*Koningin Wilhelmina Fonds*). Drs C. P. Engelfriet and H. Vos are thanked for critical reading of the manuscript.

REFERENCES

1. Hynes, R. O. (1987) *Cell 48*, 549–554.
2. Ruoslahti, E. & Pierschbacher, M. D. (1987) *Science 238*, 491–497.
3. Hemler, M. E. (1990) *Annu. Rev. Immunol. 8*, 365–400.
4. Sonnenberg, A., Janssen, H., Hogervorst, F., Calafat, J. & Hilgers, J. (1987) *J. Biol. Chem. 262*, 10376–10383.
5. Hemler, M. E., Crouse, C., Takada, Y. & Sonnenberg, A. (1988) *J. Biol. Chem. 263*, 7660–7665.
6. Sonnenberg, A., Modderman, P. W. & Hogervorst, F. (1988) *Nature 336*, 487–489.
7. Aumailley, M., Timpl, R. & Sonnenberg, A. (1990) *Exp. Cell Res. 188*, 55–60.
8. Sonnenberg, A., Linders, C. J. T., Modderman, P. W., Damsky, C. H., Aumailley, M. & Timpl, R. (1990) *J. Cell Biol. 110*, 2145–2155.
9. Gehlsen, K. R., Dillner, L., Engvall, E. & Ruoslahti, E. (1988) *Science 24*, 1228–1229.
10. Gehlsen, K. R., Dickerson, K., Argraves, W. S., Engvall, E. & Ruoslahti, E. (1989) *J. Biol. Chem. 264*, 19034–19038.
11. Wayner, E. A. & Carter, W. G. (1987) *J. Cell Biol. 105*, 1873–1884.
12. Kantor, R. R. S., Mattes, M. J., Lloyd, K. O. & Albino, A. P. (1987) *J. Biol. Chem. 262*, 15158–15165.
13. Sonnenberg, A., Hogervorst, F., Osterop, A. & Veltman, F. E. M. (1988) *J. Biol. Chem. 263*, 14030–14038.
14. Ignatius, M. J. & Reichardt, L. F. (1988) *Neuron 1*, 713–725.
15. Languino, L. R., Gehlsen, K. R., Carter, W. G., Engvall, E. & Ruoslahti, E. (1989) *J. Cell Biol. 109*, 2455–2462.
16. Elices, M. J. & Hemler, M. E. (1989) *Proc. Natl Acad. Sci. USA 86*, 9906–9910.
17. Rossino, P., Gavazzi, I., Timpl, R., Aumailley, M., Abbadini, M., Giancotti, F., Silengo, L., Marchisio, P. C. & Tarone, G. (1990) *Exp. Cell Res. 189*, 100–108.
18. Hall, D. E., Reichardt, L. F., Crowley, E., Holley, B., Moezzi, H., Sonnenberg, A. & Damsky, C. H. (1990) *J. Cell Biol. 110*, 2175–2184.
19. Lotz, M. M., Korzelius, C. A. & Mercurio, A. M. (1990) *Cell Regul. 1*, 249–257.
20. Sonnenberg, A., Linders, C. J. T., Daams, J. H. & Kennel, S. J. (1990) *J. Cell Sci. 96*, 207–217.
21. Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J. & Gipson, I. K. (1990) *Proc. Natl Acad. Sci. USA 87*, 8970–8974.
22. Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., Kennel, S. J., Aplin, J. D., Baker, J., Loizidou, M. & Garrod, D. (1991) *J. Cell Biol. 113*, 907–917.
23. Argraves, W. S., Suzuki, S., Arai, H., Thompson, K., Pierschbacher, M. D. & Ruoslahti, E. (1987) *J. Cell Biol. 105*, 1183–1190.
24. Bogaert, T., Brown, N. & Wilcox, M. (1987) *Cell 51*, 929–940.
25. Corbi, A. L., Miller, L. J., O'Connor, K., Larson, R. S. & Springer, T. A. (1987) *EMBO J. 6*, 4023–4028.
26. Corbi, A. L., Kishimoto, T. K., Miller, L. J. & Springer, T. A. (1988) *J. Biol. Chem. 263*, 12403–12411.
27. Fitzgerald, L. A., Steiner, B., Rall, S. C. Jr, Lo, S.-S. & Phillips, D. R. (1987) *J. Biol. Chem. 262*, 3936–3939.
28. Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C. Jr, Bennett, J. S. & Phillips, D. R. (1987) *Biochemistry 26*, 8158–8165.
29. Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. & Springer, T. A. (1987) *Cell 48*, 681–690.
30. Law, S. K. A., Gagnon, J., Hildreth, J. E. K., Wells, C. E., Willis, A. C. & Wong, A. J. (1987) *EMBO J. 6*, 915–919.
31. Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E. & Bennett, J. S. (1987) *J. Biol. Chem. 262*, 8476–8482.
32. Suzuki, S., Argraves, W. S., Arai, H., Languino, L. R., Pierschbacher, M. D. & Ruoslahti, E. (1987) *J. Biol. Chem. 262*, 14080–14085.
33. Suzuki, S., Huang, Z.-S. & Tanihara, H. (1990) *Proc. Natl Acad. Sci. USA 87*, 5354–5358.
34. Arnaout, M. A., Gupta, S. K., Pierce, M. W. & Tenen, D. G. (1988) *J. Cell Biol. 106*, 2153–2158.
35. DeSimone, D. W. & Hynes, R. O. (1988) *J. Biol. Chem. 263*, 5333–5340.
36. MacKrell, A. J., Blumberg, B., Haynes, S. R. & Fessler, J. H. (1988) *Proc. Natl Acad. Sci. USA 85*, 2633–2637.
37. Holers, V. M., Ruff, T. G., Parks, D. L., McDonald, J. A., Ballard, L. L. & Brown, E. J. (1989) *J. Exp. Med. 169*, 1589–1605.
38. Takada, Y. & Hemler, M. E. (1989) *J. Cell Biol. 109*, 397–407.
39. Takada, Y., Elices, M. J., Crouse, C. & Hemler, M. E. (1989) *EMBO J. 8*, 1361–1368.
40. Larson, R. S., Corbi, A. L., Berman, L. & Springer, T. (1989) *J. Cell Biol. 108*, 703–712.
41. Ignatius, M. J., Large, T. H., Houde, M., Tawil, J. W., Barton, A., Esch, F., Carbonetto, S. & Reichardt, L. F. (1990) *J. Cell Biol. 111*, 709–720.
42. Hogervorst, F., Kuikman, I., von dem Borne, A. E. G. Kr. & Sonnenberg, A. (1990) *EMBO J. 9*, 765–770.
43. Ramaswamy, H. & Hemler, M. E. (1990) *EMBO J. 9*, 1561–1568.
44. Sheppard, D., Rozzo, C., Starr, L., Quaranta, V., Erle, D. J. & Pytela, R. (1990) *J. Biol. Chem. 265*, 11502–11507.
45. Suzuki, S. & Naitoh, Y. (1990) *EMBO J. 9*, 757–763.

46. Tsuji, T., Yamamoto, F., Miura, Y., Takio, K., Titani, K., Pawar, S., Osawa, T. & Hakomori, S. (1990) *J. Biol. Chem. 265*, 7016–7021.
47. Tamaru, R. N., Rozza, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M. & Quranta, V. (1990) *J. Cell Biol. 111*, 1593–1604.
48. Hynes, R. O., Marcantonio, E. E., Stepp, M. A., Urry, L. A. & Yee, G. H. (1989) *J. Cell Biol. 109*, 409–420.
49. Barlow, J. J., Mathias, A. P., Williamson, R. & Gammack, D. B. (1963) *Biochem. Biophys. Res. Commun. 13*, 61–66.
50. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl Acad. Sci. USA 74*, 5463–5466.
51. Devereux, J., Haeberli, P. & Smithuis, O. (1984) *Nucleic Acid. Res. 12*, 387–395.
52. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
53. Geurts van Kessel, A., Tetteroo, P., von dem Borne, A., Hagemeijer, A. & Bootsma, D. (1983) *Proc. Natl Acad. Sci. USA 80*, 3748–3752.
54. Hemler, M. E., Crouse, C. & Sonnenberg, A. (1989) *J. Biol. Chem. 264*, 6529–6535.
55. Kajiji, S., Tamura, R. N. & Quaranta, V. (1989) *EMBO J. 8*, 673–680.
56. Kretsinger, R. H. (1976) *Annu. Rev. Biochem. 45*, 239–266.
57. Brown, N. H., King, D. L., Wilcox, M. & Kafatos, F. C. (1989) *Cell 59*, 185–195.
58. Corbi, A. L., Garcia-Aquilar, J. & Springer, T. A. (1990) *J. Biol. Chem. 265*, 2782–2788.
59. Heidenreich, R., Eisman, R., Surrey, S., Delgrosso, K., Bennett, J. S., Schwartz, E. & Poncz, M. (1990) *Biochemistry 29*, 1232–1244.
60. Van Kuppevelt, T. H. M. S. M., Languino, L. R., Gailit, J. O., Suzuki, S. & Ruoslahti, E. (1989) *Proc. Natl Acad. Sci. USA 86*, 5415–5418.
61. Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G. & Silengo, L. (1990) *Gene 95*, 261–266.
62. Bray, P. F., Leung, C. S. & Shuman, M. A. (1990) *J. Biol. Chem. 265*, 9587–9590.
63. Sonnenberg, A., Modderman, P. W., van der Geer, P., Aumailley, M. & Timpl, R. (1990) in *Platelet immunology: fundamental and clinical aspects* (Kaplan-Gouet, C., Schlegel, N., Salmon, Ch., McGregor, J., eds) pp. 75–95, John Libbey Eurotext, Paris, London.
64. Loftus, J. C., Plow, E. F., Jennings, L. K. & Ginsberg, M. H. (1988) *J. Biol. Chem. 263*, 11025–11028.
65. Shaw, L. M., Messier, J. M. & Mercurio, A. M. (1990) *J. Cell Biol. 110*, 2167–2174.
66. Geurts van Kessel, A., de Leeuw, B., Dekker, E. J., Rijks, L., Sheer, D., Spurr, N., Ledbetter, D., Kootwijk, E. & Vaessen, M. J. (1991) *Hum. Genet.*, in the press.
67. Sosnoski, D. M., Emanuel, B. S., Hawkins, A. L., van Tuinen, P., Ledbetter, D. H., Nussbaum, R. L., Kaos, F.-T., Schwartz, E., Phillips, D., Bennet, J. S., Fitzgerald, L. A. & Poncz, M. (1988) *J. Clin. Invest. 81*, 1993–1998.
68. Rettig, W. J., Dracopoli, N. C., Goetzger, T. A., Spengler, B. A., Biedler, J. L., Oettgen, H. F. & Old, L. J. (1984) *Proc. Natl Acad. Sci. USA 81*, 6437–6441.

Sequence and Tissue Distribution of the Integrin α9 Subunit, a Novel Partner of β1 That Is Widely Distributed in Epithelia and Muscle

Elise L. Palmer, Curzio Rüegg, Ronald Ferrando, Robert Pytela,* and Dean Sheppard

The Lung Biology Center, the Cardiovascular Research Institute, the Center for Occupational and Environmental Health, and the Departments of Medicine and *Pharmacology, University of California, San Francisco, California 94143

Abstract. The integrin family of adhesion receptors consists of several heterodimeric glycoproteins, each composed of one α and one β subunit. A novel integrin α subunit partial cDNA isolated from TGF-β stimulated guinea pig airway epithelial cells has previously been reported (Erle, D. J., D. Sheppard, J. Bruess, C. Rüegg, and R. Pytela. 1991. *Am. J. Respir. Cell Mol. Biol.* 5:170–177). We have now determined cDNA and amino acid sequence for the human homolog of this subunit, named α9, from a human lung cDNA library, a human small intestine cDNA library, and cDNA from the cell lines U937, HL-60 and Tera-2. This sequence is predicted to encode a 1006-amino acid mature protein that shares 39% identity with the previously identified integrin subunit α4. By Northern blot analysis, α9 mRNA was detected in the human carcinoma cell lines Tera-2 and Caco-2. Anti-peptide antibodies against the predicted COOH-terminal sequence of α9 immunoprecipitated a heterodimer (140 kD/115 kD nonreduced; 150 kD/130 kD reduced) from Tera-2 lysates. Immunodepletion of β1-containing integrins with Tera-2 lysates removed α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. α9 was detected by immunohistochemistry in airway epithelium, in the basal layer of squamous epithelium, and in smooth muscle, skeletal muscle, and hepatocytes.

THE integrins are a large family of cell surface glycoproteins that mediate cell–cell and cell–matrix adhesion (21). All known members of this family are heterodimers consisting of an α and a β subunit that bind noncovalently to each other. Published reports suggest the existence of 8 β subunits (β1–β8) (2, 12, 14, 20, 26, 30, 32, 34, 37, 40, 41) and 16 α subunits (2–5, 9, 10, 13, 16, 23, 25, 28, 33, 38, 39, 42–47). 13 of these have been completely sequenced (α1–α8, αv, αM, αL, αX, αIIb). The existence of αIEL and αLRI has been established on the protein level. We have previously reported identification of partial integrin α subunit cDNA sequences using degenerate oligonucleotide primer pairs and the PCR (13). Primers were designed based on two highly conserved regions, separated by 72–92–amino acid residues, located within the fifth and sixth repeated domains of the previously reported integrin α subunits. PCR amplification of cDNA using these primers resulted in the identification of several integrin α subunit partial cDNAs. From guinea pig airway epithelial cells six different sequences were identified. Five of these were 88 to 92% identical to the sequences of α1–α3, α5 and αv from other species and were presumed to encode the guinea pig homologs of those subunits. One of the amplified sequences was only 24–60% identical to previously reported α subunits. This novel cDNA was predicted to encode a 71-amino acid fragment of an integrin α subunit, provisionally designated αA. We now report the predicted coding sequence of the human homolog of this novel subunit. We have identified mRNA encoding this subunit and the corresponding protein in two cell lines, including the human teratoma cell line Tera-2, and demonstrate that this α subunit associates principally with the integrin β1 subunit in these cells. Based on the convention of sequentially numbering newly identified partners of β1, we have called this α subunit α9. We also demonstrate wide distribution of α9 in epithelia, smooth muscle, skeletal muscle, and hepatocytes.

Materials and Methods

Cell Lines, RNA Purification, and cDNA Synthesis

ATCC human cell lines were maintained in DME (HeLa, Tera-2, Caco-2,) or RPMI 1640 media (U937, HL-60, Raji, HuT 78, MOLT-4) containing 10% FBS. Media were obtained from BioWhittaker, Inc. (Walkersville, MD).

mRNA was isolated from resected human lung tissue using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Total cellular RNA

Address all correspondence to Dr. Dean Sheppard, Lung Biology Center, UC Box 0854, San Francisco, CA 94143.

was isolated using the LiCl/Urea method (12). Single-stranded cDNA was synthesized at 44°C for 1 h from 1 to 5 μg of mRNA or 20–40 μg of total RNA using the Superscript cDNA Synthesis System (GIBCO-BRL, Gaithersburg, MD) and random DNA hexamers. In some cases cDNA was synthesized as above using a previously described sequence tagged random DNA hexamer primer (CGAGGGGGATGGTCGACGGAAGCGACC-NNNNNNT) (15).

Amplification and Purification of Lambda Library DNA

Aliquots of λgt11 libraries containing 1–5 million plaque forming units were mixed with 500 μl of LE392 bacteria (OD = 0.5) in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCl, pH 7.0, 0.01% gelatin), incubated 15 min at 37°C, and shaken at 250 rpm overnight at 37°C in Luria broth containing 10 mM $MgCl_2$ and 0.2% maltose. The cultures were incubated at 37°C and 250 rpm for 30 min with 5% chloroform. Aliquots of the aqueous layer were subjected to three cycles of 3 min in dry ice/ethanol followed by 3 min at 90°C, phenol/chloroform extraction, and precipitation with ethanol.

PCR Amplification

PCR reactions were performed in 25–200 μl reaction volumes and contained 1 × *Taq* buffer (Promega, Corp., Madison, WI) with 1.5 mM $MgCl_2$, 0.1 μM each of two primers, 0.025 U/μl *Taq* Polymerase (Promega Corp.), 1–8 μl of DNA template, and 0.1 mM each of dATP, dGTP, dCTP, and dTTP. Reactions were heated to 94°C for 4 min in a thermocycler (ERICOMP, Inc., San Diego, CA) and then subjected to 30 cycles of PCR followed by 10 min at 72°C. Reactions that contained degenerate oligonucleotide primers were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 48 or 53°C, and 45 s at 72°C. Reactions that contained α9-specific primer pairs designed to amplify DNA fragments less than 750 nucleotides were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 57°C, and 60 s at 72°C. Reactions that contained an α9-specific primer, a λgt11-specific primer (λgt11F: CCTCCGTCGACGGTGGCGACGACTCCTGGAGCCCG or λgt11R: CCTCCGTCGACTTGACACCAGACCAACTGGTAATG), and purified concentrated total library DNA were subjected to PCR cycles consisting of 45 s at 94°C, 45 s at 60°C, and 120 s at 72°C. All other PCR reactions were subjected to cycles consisting of 45 s at 94°C, 45 s at 57°C, and 120 s at 72°C. Products of each PCR reaction were analyzed by agarose gel electrophoresis using standard or low gel temperature agarose.

PCR reactions designed to amplify 3′ sequence from sequence tagged cDNA, described above, contained α9-specific forward primers and primers complementary to the sequence tagged random hexamer (GATGGTCGACGGAAGCGAAC or CGAGGGGGATGGTCGACGG) and were performed as above.

Cloning of DNA Fragments

Restriction-digested DNA fragments were isolated on low gel temperature agarose and purified by phenol/chloroform extraction and ethanol precipitation. Fragments were ligated into restriction-digested, dephosphorylated pBluescript vector (Stratagene, La Jolla, CA) with T4 DNA ligase (GIBCO-BRL). The ligation mixture was used to transform competent *Escherichia coli* (JM-109; Clontech, Palo Alto, CA). Selected plasmids were purified from liquid cultures using the Pharmacia miniprep lysis kit (Pharmacia Fine Chemicals/LKB, Pleasant Hill, CA) and sequenced using Sequenase 2.0 (United States Biochemical Corporation, Cleveland, OH), ^{35}S-dATP (Amersham Corp., Arlington Heights, IL), T3 primer, T7 primer, and a variety of primers specific for α9.

Library Screening

Partial cDNA fragments were used as templates for the random-primed synthesis of [^{32}P]dCTP-labeled probes (multiprime DNA labeling system; Amersham Corp.). These probes were used to screen an oligo-dT-primed λgt11 cDNA library from human lung tissue including trachea and bronchioles (catalog No. HL 1066b; Clontech) and a mixed random and oligo-dT primed λgt11 cDNA library from human small intestine tissue (catalog No. HL1133b; Clontech). Hybridizations were performed at 50°C for 16 h in hybridization buffer (40% formamide, 50 mM sodium phosphate, pH 6.5, 800 mM NaCl, 0.05% polyvinylpyrrolidone, 0.05% BSA, 0.05% ficoll, 1 mM EDTA, 0.1% SDS, and 10 ng/ml heat-denatured, sonicated salmon sperm DNA). Filters were washed twice in 1× SSC containing 0.1% SDS for 5 min at room temperature, and once in 0.5× SSC containing 0.1% SDS for 1 h at 50°C and exposed to film for 18 h at −80°C with intensifying screens. Positive library clones were isolated by further rounds of screening, and the inserts were isolated either by PCR amplification using λgt11-specific primers or by EcoRI digestion of purified phage DNA. The inserts were then subcloned into EcoRI-digested pBluescript and sequenced.

Northern Blot Analysis

Total cellular RNA was electrophoresed through a formamide–agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Corp.). Radiolabeled probe was synthesized from 2.1 kb of α9 sequence using the Multiprime Labeling System (Amersham Corp.) and [^{32}P]dCTP. Filters were incubated at 50°C for 16 h in hybridization buffer containing 5× SSC, 40% formamide, 20 mM Tris, pH 7.5, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% ficoll, 10% dextran sulfate, and 100 μg/ml heat-denatured, sonicated salmon sperm DNA, and washed in 5× SSC containing 0.1% SDS at 50°C for 30 min. After washing, filters were exposed to film at −80°C with an intensifying screen.

Antibodies and Immunoprecipitations

Polyclonal antiserum was generated against the α9 peptide CRKENEDSW-DWVQKQ. Peptide synthesis, conjugation to KLH, and injection of rabbits was performed by Immunodynamics, Inc. (La Jolla, CA). α9 antibodies were affinity purified from crude antiserum on a peptide-lysozyme-Affigel column as follows. Affigel-10 slurry (4-ml bed volume) (Bio Rad Labs., Richmond, CA) was washed three times with cold 10 mM sodium acetate, pH 4.5; rinsed once with 0.1 M potassium phosphate, pH 7.5; mixed with 100 mg lysozyme (Sigma Immunochemicals, St. Louis, MO) in 8 ml 0.1 M potassium phosphate, pH 7.5, for 2 h at room temperature; washed twice with 0.1 M potassium phosphate, pH 7.5; mixed with 8 ml 0.2 M ethanolamine, pH 8.0, for 2 h at room temperature; and washed three times with PBS. Lysozyme-Affigel (600-μl bed volume) was washed with 0.05 M sodium phosphate, pH 8.0; mixed with 2.5 mg sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemicals, Rockford, IL) in 1 ml 0.05 M sodium phosphate, pH 8.0, for 30 min at room temperature; washed with 0.05 M sodium phosphate; pH 8.0; mixed with 3 mg α9 peptide in 500 μl 0.05 M sodium phosphate, pH 8.0, for 3 h at room temperature; and washed with (*a*) 0.05 M sodium phosphate, pH 7.0, (*b*) 200 mM glycine, pH 1.5, (*c*) 0.05 M sodium phosphate, pH 7.0, (*d*) 8 M urea, and finally (*e*) 0.05 M sodium phosphate, pH 7.0. 5–10-ml aliquots of antiserum were mixed with the peptide-lysozyme-Affigel overnight at 4°C. The slurry was transferred to a column and washed with 0.05 M sodium phosphate, pH 7.0, until the OD 280 of the wash buffer was less than 0.01. Antibodies were eluted in 1.25-ml fractions with 200 mM glycine, pH 1.5, and collected in tubes containing 250 μl 1 M sodium phosphate, pH 8.0. Pooled fractions were dialyzed overnight at 4°C against PBS containing 0.02% azide.

mAb P5D2 (11) directed against the β1 integrin subunit was a gift from Elizabeth Wayner (University of Minnesota, Minneapolis, MN). mAb R6G9 directed against the β6 integrin subunit was generated in our laboratory. For some experiments antibodies were cross linked to protein A–Sepharose with dimethylpimelimidate (17).

Cells were surface labeled with [^{125}I], lysed in immunoprecipitation buffer (100 mM Tris–HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 0.1% NP-40, and 300 mM NaCl), and immunoprecipitated by standard techniques. Samples were analyzed by SDS-PAGE on 7.5% acrylamide gels and exposed to film at −80°C with intensifying screens.

Tissue Staining

Frozen sections (5 μm) of tissue were fixed in either 2% paraformaldehyde (mouse tissue) at room temperature or in −18°C acetone (human tissue) for 5 min and subsequently rinsed in PBS. Sections were blocked for endogenous peroxidase activity with Peroxoblock Solution (Zymed Laboratories, Inc., South San Francisco, CA) for 45 s at room temperature. After rinsing, sections were preblocked with 0.5% casein/0.05% thimerosal/PBS for 15 min at room temperature and then incubated overnight at 4°C in primary antibody (1:200 in 0.5% casein/0.05% thimerosal/PBS) that either was or was not preincubated with 0.1 mg/ml α9 peptide for 30 min at 4°C. After rinsing in PBS, sections were incubated in either biotinylated donkey anti–rabbit secondary antibody (Amersham Corp.) at a dilution of 1:200 or peroxidase-conjugated goat anti–rabbit secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:250 for 1 h at room temperature in 0.5% casein/0.05% thimerosal/PBS. Sections incubated with biotinylated secondary antibodies were then rinsed and incubated in ABC avidin/peroxidase reagent (Vector Laboratories) for 1 h at room temperature. Chromagen

was developed using the DAB Plus Kit from Zymed Laboratories. Reactions were monitored until suitable color development was achieved. The signal was enhanced with 0.5% nickel chloride, and the sections were rinsed in distilled water. Sections were subsequently air dried and then mounted with Permount (Fisher Scientific, Pittsburgh, PA) onto clean slides.

Protein Sequencing

Human uterine tissue was homogenized in immunoprecipitation buffer and cleared by centrifugation at 200 *g*. The supernatant was incubated with affinity-purified α9 anti-cytoplasmic peptide antiserum cross-linked to protein A-Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) overnight at 4°C. The beads were then washed extensively with immunoprecipitation buffer and heated to 95°C for 5 min in 2% SDS, 300 mM 2-mercaptoethanol, 80 mM Tris, pH 6.8. The supernatant was concentrated on a Centricon-10 Concentrator (Amicon, Beverly, MA), subjected to 7.5% SDS-PAGE, transferred to PVDF membrane (Bio Rad Labs. Hercules, CA) for 3 h at 50 V in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11/10% methanol, and quantified by staining with 0.1% Coomassie blue R-250 in 40% methanol/1% acetic acid. A prominent band at 150 kD was microsequenced by the USCF Biomedical Resource Center (San Francisco, CA).

Results

Cloning and Sequencing of Human α9

A novel 223-nucleotide partial guinea pig integrin α subunit cDNA (αA) was previously reported by Erle et al. (13). To obtain the corresponding human sequence of the novel subunit, we used the previously described degenerate integrin α subunit forward primer (A14F: CGGAATTCGGIG-A(AG)CAG(AC)TIG(CG)I-(GT)CIGA(CT)TT(CT)GG), a degenerate reverse primer based on the αA sequence (AN2R: CAAGTCGACAA(AG)TGIGC(AG)TT(GC)TA-IGCIC(GT)(AG)TC), and cDNA made from human lung tissue. A band of the predicted size (204 nucleotides) was obtained (data not shown), subcloned into pBluescript, and sequenced. The resulting nucleotide sequence was 88% identical to the sequence of guinea pig αA and 39–54% identical to other known human integrin α subunits. For reasons discussed later, we have named this novel human integrin subunit α9.

The 204-nucleotide α9 cDNA fragment was used to screen a human lung cDNA library. One hybridizing clone, L1 (Fig. 1), was identified, purified, subcloned and sequenced. This 1,678-bp clone contained a 1,123-nucleotide open reading frame that included the probe sequence.

We were able to amplify α9 from cDNA from leukocyte cell lines U937 (clones U1–U3) and HL-60 (H1) by PCR using α9-specific primers. To isolate additional sequence from these cell lines, we used a previously described 3'-sequence extension technique (15) which employs PCR. One clone from HL-60 cDNA, H2, and one clone from U937 cDNA, U4, obtained by this method contained 117 nucleotides of α9 sequence.

PCR was performed on amplified purified human lung cDNA library DNA using α9-specific forward oligonucleotide primers and λgt11-specific primers (λgt11F and λgt11R). Individual bands were isolated, reamplified, subcloned, and sequenced. One clone, L2a, consisted of 300 nucleotides of α9 sequence. Clone L2a was used as a probe for screening the human lung library and one hybridizing clone, L2b, was isolated, purified, subcloned, and sequenced. Both clone L2a and clone L2b ended at nucleotide 1817, suggesting that clones L2a and L2b represented the same library clone.

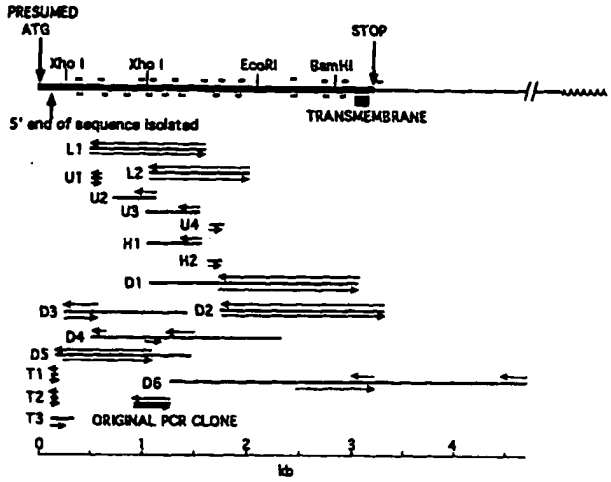


Figure 1. Map of α9 sequencing strategy. Shown are the location of clones used to obtain the partial sequence of human α9. Internal sequence was obtained by use of specific α9 sequencing primers (*dashes*) and by generating restriction fragments at sites shown. The clone labeled *original PCR clone* was obtained from human lung cDNA using homology based PCR. Clones labeled *L1* and *L2* were obtained from a human lung cDNA library. Clones labeled *U1–U4* were obtained by PCR from U937 cDNA. Clones *H1* and *H2* were obtained by PCR from HL-60 cDNA. Clones labeled *D1–D6* were obtained from a human small intestine cDNA library. Clones labeled *T1–T3* were obtained by PCR from Tera-2 cDNA. The direction and extent of sequencing are indicated with arrows. All reported sequence was sequenced completely in both directions from at least two independent clones. Noncoding sequences found on the 5' end of clones *L1* (555 bp), D3 (108 bp), and *D6* (~1200 bp) are not indicated on the map.

We screened a human duodenal cDNA library with probes from clones L1 and L2. 12 independent hybridizing clones were identified, isolated, subcloned, and analyzed. Six of these were determined to contain additional α9 cDNA and were sequenced (D1–D6). D2 and D6 contained a predicted transmembrane domain, cytoplasmic domain, and translation stop codon. D6 also had an additional 1.1 kb, 3' of the stop codon, which did not extend to the poly(A) tail.

To complete the α9 sequence, α9 protein was purified from human uterus using anti-α9 antiserum, described later, and microsequenced. The amino terminal sequence was equivocally determined to be YNLD(T/P)(Q/E). A series of degenerate forward oligonucleotide PCR primers were designed based upon these possible amino-terminal sequences. PCR amplifications were performed using each of the degenerate forward primers paired with α9-specific reverse oligonucleotide primers and Tera-2 cDNA (data not shown). Reactions using a degenerate forward primer based upon the sequence YNLDPQ and two different α9-specific reverse primers resulted in intense bands which were cloned and sequenced (T1–T3).

We have sequenced 3,139 nucleotides (Fig. 2) of α9 cDNA that contains a 3,000-nucleotide open reading frame that includes a termination signal but lacks an initiation codon. Based on this cDNA sequence and the amino terminal sequence we obtained, we predict the mature α9 protein to be 1,006 amino acids in length with a 947–amino acid extracellular domain, a 26–amino acid transmembrane domain, and

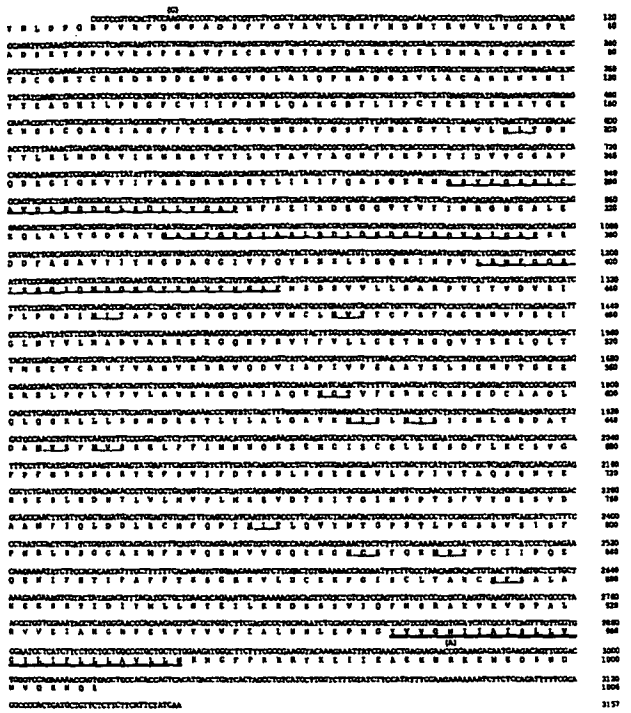


Figure 2. Human integrin α9 cDNA sequence and deduced amino acid sequence. The amino acid sequence is shown in one-letter code below the first nucleotide of each codon. The putative transmembrane domain is shown by a double underline. Asparagine residues (*N*) that are potential sites for N-glycosylation are indicated by dotted underline. Conserved putative metal binding domain are indicated by a single underline. Nucleotide 36 was determined to be A from some clones and G from others (indicated by a *G* in parentheses above the nucleotide location). Nucleotide 2974 was C in some clones and A in others. None of these nucleotide variations changed the deduced amino acid sequence. This sequence data is available from EMBL/GenBank/DDBJ under accession number L24158.

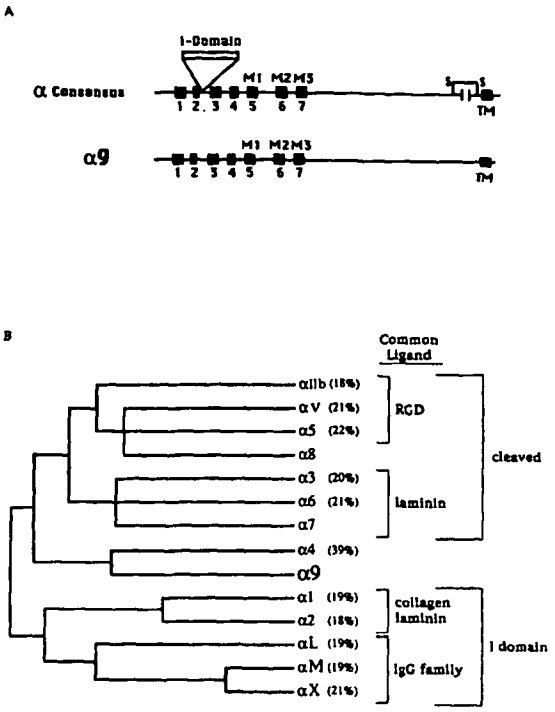


Figure 3. Comparison of α9 to other human integrin α subunits. (*A*) The predicted structure of α9 is shown schematically compared to the consensus structure of α subunits, with the NH_2-termini on the left. Conserved sequence repeats are indicated by black boxes numbered 1-7. The three conserved repeats that represent complete putative metal binding domains are labeled *M1-M3*. The I domain present in some subunits is indicated between conserved repeats 2 and 3. The cleavage site present in some subunits is indicated near the transmembrane domain by *S-S*. The transmembrane domain is indicated with *TM*. (*B*) The sequence similarity between all the previously reported human integrin α subunits is indicated by tree diagram (reference 21). The percent amino acid identity between α9 and each human integrin α subunit for which the sequence has been published in indicated parenthetically next to that subunit. Three subfamilies can be differentiated based on structural and functional features that correlate with sequence homologies: (*a*) subunits which are cleaved into two disulfide-linked fragments are involved in binding to the long arm of laminin or binding to RGD sequences present in certain extracellular matrix proteins, (*b*) subunits which contain the I domain are involved in binding to collagen and the cross region of laminin or to IgG superfamily members, and (*c*) other subunits (α4 and α9) which neither exhibit the conserved cleavage pattern nor contain the I domain.

a 33-amino acid cytoplasmic domain. α9 has 12 asparagine residues that are potential N-glycosylation sites (NXT/S).

The Relationship of α9 to Other Integrin α Subunits

Comparison of α9 to the general structure of integrin α subunits is indicated in Fig. 3 *A*. The consensus structure includes: a large NH_2 terminal extracellular domain containing seven conserved repeats (four partial and three complete putative metal binding domains) and in some cases the I domain, an insertion of approximately 200 amino acids, a single transmembrane-spanning domain, and a short COOH-terminal cytoplasmic domain. Some subunits are cleaved into two disulfide-linked fragments near the transmembrane domain (18). α9 contains the seven conserved repeats found in all known integrin α subunits. α9 does not contain an I domain nor is it cleaved. The cytoplasmic domain of α9, as with all α subunits, contains the highly conserved sequence GFF(R/K)R.

Sequence, structural, and functional relationships between the integrin α subunits are shown in Fig. 3 *B*. The integrin α subunit family has three distinct subfamilies. The first subfamily consists of the seven α subunits that undergo cleavage near the transmembrane domain. The second subfamily includes the five α subunits that contain an I domain. The third subfamily consists of α4, which contains neither the I domain nor undergoes cleavage yielding disulfide-linked fragments. The deduced partial amino acid sequence of α9 is 39% identical to the integrin α4 subunit sequence and 18-22% identical to the other known human integrin α subunit sequences. Based on its sequence and structural similarity to α4, α9 is clearly a member of the third integrin α subunit subfamily.

Fig. 4 shows the alignment of the predicted α9 amino acid sequence with α4. All 23 cysteine residues found in α9 align with those found in α4 (α4 contains one additional cysteine),

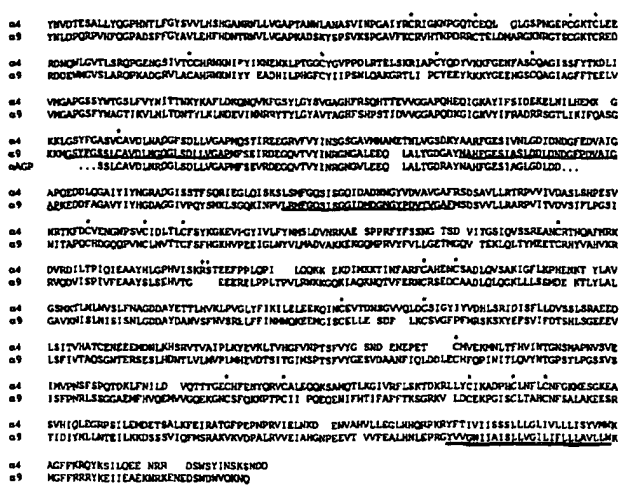


Figure 4. Alignment of α9 with its closest integrin α subunit relative, α4. The deduced amino acid sequences of human α9 and guinea pig αA (*reference* 13) and the previously reported sequence of the mature form of α4 (*reference* 46) are shown aligned using the one-letter amino acid code. Cysteine residues conserved between α9 and α4 are indicated by an asterisk (★). The conserved metal binding repeats common to all integrin α subunits are underlined. The predicted transmembrane domains are indicated by double underline. The cleavage site in α4 (++), between Arg558 and Ser559, is not present in α9.

including the 19 cysteines generally found in integrin α subunits. α4 contains a cleavage site following Lys557-Arg558 which is absent in α9.

α9 Is Expressed by Teratoma and Colon Carcinoma Cells and Forms a Heterodimer with β1

To demonstrate expression of α9 mRNA and to identify cell lines that express α9, Northern blots were performed. Total RNA was isolated from a variety of cell lines and analyzed by Northern blotting with a 2.1-kb α9 cDNA probe (Fig. 5). This probe hybridized with an ~7.0-kb mRNA band (size markers not shown) that was present in two of the cell lines tested, i.e., Tera-2, and Caco-2 cells (lanes *1* and *2*). The smaller band may represent partially degraded α9 mRNA. Alternatively, a second α9 mRNA species derived by alternative splicing or an alternative polyadenylation signal may be present in these cells. Tera-2 cells are derived from an embryonal carcinoma, and Caco-2 cells from a colon carcinoma. The carcinoma-derived cell line HeLa (lane *3*) and the leukocyte-derived cell lines Raji, HuT 78, MOLT-4, and HL-60 (lanes *4–7*) were negative for expression of α9, as were cell lines U937 (histiocytic lymphoma), K-562 (chronic myelogenous leukemia), Daudi (Burkitt's lymphoma), AN3CA (endometrial carcinoma), and JEG-3 (choriocarcinoma) (data not shown).

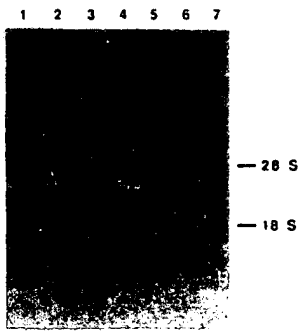


Figure 5. Identification of α9 mRNA by Northern blot analysis. Total RNA (30–40 μg/lane) isolated from human cell lines Caco-2 (lane *1*), Tera-2 (lane *2*), HeLa (lane *3*), Raji (lane *4*), HuT 78 (lane *5*), MOLT-4 (lane *6*), and HL-60 (lane *7*) was probed for α9 mRNA using 2.1 kb of ^{32}P-labelled α9 cDNA. The positions of the 28- and 18-S ribosomal bands are indicated on the left.

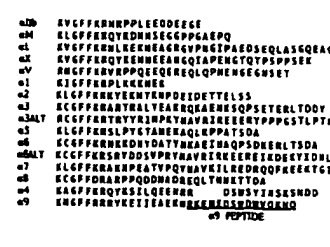


Figure 6. Alignment of integrin α subunit cytoplasmic domains. The COOH-terminal cytoplasmic sequences of the known human integrin α subunits are shown (*references* 2, 5, 9, 10, 28, 33, 39, 42–45). The amino acid sequence of the peptide used for production of α9-specific rabbit antiserum is underlined and labeled α9 *Peptide.*

The sequences of the cytoplasmic domains of the known vertebrate integrin α subunits are shown in Fig. 6. It is evident that the α9 cytoplasmic domain is distinct from all other subunits, except for very limited similarity to the α4 cytoplasmic domain. A 15-amino acid α9 peptide (underlined in Fig. 6) with an additional cysteine residue at the NH_2 terminus was used to generate polyclonal rabbit antiserum which was subsequently affinity purified on the same peptide coupled to Sepharose. The sequence of this peptide is distinct from the cytoplasmic domain sequences of the other α subunits, except for a three-amino acid identity (DSW) with α4. Cross-reactivity of the anti-α9 peptide serum with α4 or any other known α subunit is unlikely.

Immunoprecipitations were performed on surface labeled Tera-2 lysate with α9 antiserum or with a MAb against the integrin β1 subunit. The immunoprecipitates were analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 7). Immunoprecipitation with anti-β1 yielded major

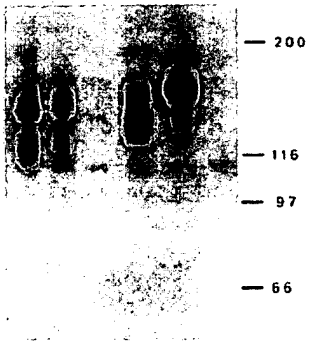


Figure 7. Immunoprecipitation of α9- and β1-containing integrins from lysates of Tera-2 cells. Aliquots of ^{125}I-surface-labeled lysates from Tera-2 cells were immunoprecipitated with a monoclonal anti-β1 antibody (lanes *1* and *4*), affinity purified polyclonal antiserum raised against a portion of the cytoplasmic domain of α9 (lanes *2* and *5*), and preimmune serum (lanes *3* and *6*). The proteins were analyzed by SDS-PAGE under nonreducing (lanes *1–3*) and reducing (lanes *4–6*) conditions followed by autoradiography. The positions of molecular size markers (in kD) are shown to the right. 10-fold less lysate was used in the β1 immunoprecipitation than in the α9 or preimmune serum immunoprecipitations.

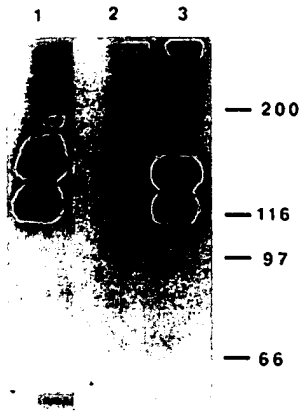


Figure 8. Immunodepletion of β1 containing integrins. Aliquots of ^{125}I-surface-labeled lysates from Tera-2 cells were subjected to four rounds of immunoprecipitation with an anti-β1 monoclonal antibody or an anti-β6 mAb cross-linked to protein A-Sepharose. The resulting supernatants were immunoprecipitated with affinity purified α9 polyclonal antiserum. The immunoprecipitated material (anti-β1 first round immunoprecipitate, lane *1*; anti-β1 followed by α9 antiserum, lane *2*; anti-β6 followed by α9 antiserum, lane *3*) was analyzed by SDS-PAGE under nonreducing conditions followed by autoradiography. The positions of molecular size markers (in kD) are shown to the right.

nonreduced bands at 140 and 115 kD and a minor band at 180 kD (lane *1*). These bands changed upon reduction to 190, 150, and 130 kD (lane *4*). α9 antiserum immunoprecipitated a heterodimer consisting of 140 and 115 kD subunits nonreduced (lane *2*) and 150 and 130 kD subunits reduced (lane *5*). The upper band migrated with the same apparent molecular mass as several other previously reported integrin α subunits (19). The lower band migrated with the same apparent molecular mass as β1 (19). The band present in α9 immunoprecipitations at 110 kD is also present in immunoprecipitations using preimmune serum (lanes *3* and *6*), and hence is nonspecific.

To determine if β1 was a β subunit partner for α9, Tera-2 lysates were subjected to four rounds of immunodepletion with either anti-β1 or anti-β6 antibody followed by immunoprecipitation with affinity purified α9 antiserum. The results (Fig. 8) show that α9 could not be immunoprecipitated from lysate depleted of β1 (lane *2*). Immunodepletion with the anti-β6 antibody (lane *3*) did not interfere with subsequent precipitation of α9, demonstrating that nonspecific loss of α9 does not occur during multiple rounds of immunodepletion. These results suggest that β1 is the predominant β subunit partner for α9 in Tera-2 cells. This justifies our terminology, following the convention of sequentially numbering the α subunit partners of β1.

α9 Is Widely Distributed in Normal Tissues

Because α9 was initially isolated from airway epithelial cell cDNA, we attempted to identify α9 in human airway tissue by immunohistochemistry. Fig. 9 *A*, shows a human airway biopsy section, including epithelium and submucosa, stained with hematoxylin. α9 antiserum intensely stained the airway epithelium (*B*). When the antiserum was incubated with α9 peptide prior to tissue staining, staining of the epithelium was substantially blocked (*C*).

Because we were able to detect α9 immunoreactivity in airway tissue, we examined the expression of α9 in other tissues. Sections of mouse tissue were stained with hematoxylin (Fig. 9, *D*, *G*, and *J*) α9 antiserum (*E*, *H*, and *K*), or α9 antiserum preblocked with α9 peptide (*F*, *I*, and *L*). In mouse esophagus (*D–F*), the basal layer of the epithelium (*arrow*) was stained by α9 antiserum. Skeletal muscle (*M*) present in this section also showed α9 immunoreactivity. In mouse small intestine (*G–I*) both the longitudinal and circumferential smooth muscle layers (*SM*) reacted with the α9 antiserum. Hepatocytes in the liver (*J*, *K*, and *L*) demonstrate cell surface localization of α9. A variety of other mouse tissues were analyzed for the presence of α9. Table I summarizes these results. The tracheal epithelium; basal epithelium of the larynx, pharynx, esophagus, skin, and cornea; smooth muscle of trachea, veins, duodenum, colon, stomach, and esophagus; skeletal muscle; hepatocytes; and splenic giant cells all demonstrated α9 immunoreactivity. In all of these cases, staining was blocked by incubation of the α9 antiserum with α9 peptide prior to tissue staining.

Discussion

This report presents three novel findings. First, we present the complete amino acid sequence, deduced from cDNA and amino terminal sequencing, of a new member of the human integrin α subunit family, α9. Second, we demonstrate that α9 forms an integrin heterodimer with the known β subunit, β1. Third, we show that α9 is expressed in a variety of cell types in vivo including airway epithelial cells, the basal layers of squamous epithelium, smooth muscle, skeletal muscle, and hepatocytes.

Comparison of the deduced amino acid sequence of α9 with the previously published sequences of human integrin α subunits clearly demonstrates that this protein is a member of the integrin α subunit family. α9 has high sequence homology with other human integrin α subunits and has predicted structural features common to the integrin α subunits including (*a*) a large extracellular domain containing four partial and three complete metal binding domains, 19 consensus cysteine residues, and several potential N-glycosylation sites, (*b*) a single transmembrane spanning domain, and (*c*) a short cytoplasmic domain containing the sequence GFF(K/R)R. Although α9 is the only human integrin α subunit to have the alternative sequence GFFRR, chicken α3 contains GFFRR (22). Both variations of this sequence are also found in the DNA binding domain of the members of the steroid hormone receptor superfamily (29). The functional significance of this sequence is not known, although it has been reported that a GFFKR-containing peptide can interact with the Ro/SS-A antigen (calreticulin) (35).

There are reports of four other integrin α subunits for which the human sequence has not been published. We do not believe that any of these four are identical to the subunit we describe based on the following evidence. The integrin subunits α7 and α8 have been cloned from other species, and

Figure 9. Immunohistochemical localization of α9. Frozen sections of human airway biopsies (*A–C*) and mouse esophagus (*D–F*), duodenum (*G–I*), and liver (*J–L*) were stained with hematoxylin (*A*, *D*, *G*, *J*), with α9 antiserum (*B*, *E*, *H*, *K*), or with α9 antiserum preincubated with α9 peptide (*C*, *F*, *I*, *L*). The α9 antiserum specifically stained airway epithelium, esophageal skeletal muscle (*M*) and basal cells of the squamous epithelium (*arrow*), duodenal smooth muscle (*SM*), and hepatocytes.

Table I. Immunohistochemical Localization of α9 in Mouse Tissue

Tissue	α9 expression	Distribution
Trachea	+	Epithelium, smooth muscle
Larynx	+	Basal epithelium
Esophagus	+	Basal epithelium, smooth muscle
Skeletal muscle	+	
Stomach	+	Smooth muscle
Duodenum	+	Smooth muscle
Skin	+	Basal keratinocytes
Cornea	+	Basal epithelium
Veins	−	Smooth muscle
Aorta	−	
Pancreas	+	
Liver	−	Hepatocytes
Heart	+	
Spleen		Giant cells only (rare)

the resulting sequences have less than 21% amino acid identity to α9 (4, 38). Partial cDNA sequences of the human homologs of α7 and α8 are clearly distinct from the α9 sequence (our unpublished observations). Both of the subunits of a novel integrin expressed on activated leukocytes, termed the leukocyte response integrin, have different electrophoretic mobilities than does α9 (16). The previously identified HML-1 antigen is an integrin composed of a novel α subunit, αIEL, paired with β7 (7, 25, 47). The HML-1 antigen has been detected in intraepithelial lymphocytes and a subset of lymphocytes located in the submucosal layer of the intestine (8), whereas α9 was not detected in either the intestinal epithelial layer or submucosa.

We screened several cell lines by Northern blotting for expression of α9 and identified two cell lines, Tera-2 and Caco-2, that expressed significant levels of α9 mRNA. Immunoprecipitation of lysates from these cell lines with α9 anti-peptide antiserum precipitated a heterodimer consisting of a larger subunit of similar size to many other α subunits and a smaller subunit that comigrated with β1 (Caco-2 data not shown). Immunodepletion of β1 from Tera-2 lysates removed all detectable α9 immunoreactivity, suggesting that β1 is the principal β subunit partner for α9 in these cells. This increases the number of α subunits known to associate with β1 to 10. Three of these α subunits (αv, α4, and α6) can also form heterodimers with other β subunits. We cannot exclude the possibility that α9 is expressed in low abundance with another β subunit in Tera-2 cells or that α9 has other β subunit partners in other cell lines or in vivo.

The closest known relative of the α9 subunit is the α4 subunit. α9 and α4 share 39% amino acid identity and are both equally divergent from the other known α subunits. Consistent with their similarity, α9, like α4, is not composed of two disulfide-linked fragments and does not contain an I domain. Integrin α subunits generally demonstrate low correlation of sequence similarity with tissue distribution. Therefore, even though α9 and α4 are closely related, we would not necessarily expect them to have similar expression patterns. α4 was first identified on leukocytes (19) and has more recently been shown to be expressed on endothelial cells (31) and in developing, but not adult, skeletal muscle (36). In contrast, α9 was detected in adult skeletal and smooth muscle and in a subset of epithelial cells and is not generally expressed in lymphocytes or in endothelium. Although α9 was detected by PCR in two leukocyte cell lines, it could not be detected by Northern blotting in these cells. The overwhelming majority of lymphocytes present in sections of mouse spleen did not stain with the α9 antibody. However, rare, splenic giant cells did demonstrate α9 immunoreactivity, suggesting that α9 may be expressed in at least some leukocytes in vivo.

Our results show widespread expression of α9 in vivo. In airway epithelium and the basal layer of squamous epithelium, α9 appears to be distributed at cell–cell borders as well as at cellular contacts with basement membrane. In smooth muscle, skeletal muscle, and hepatocytes, α9 appears to be uniformly distributed over the cell surface. In these tissues, α9 is expressed diffusely at sites of homotypic cell to cell contact in cells that are not actively involved in spreading, migration, or any other obvious dynamic interaction with the extracellular matrix. These results suggest that α9 may be involved in homotypic cell–cell interactions. Although integrins are not generally thought to be involved in homotypic cell–cell adhesion in non-leukocyte cells, localization of integrins to cell–cell contacts has been shown for α2β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and endothelial cells (27); for α3β1 in bronchial epithelial cells (1), cultured keratinocytes (6), and a variety of transformed cell lines (24); and for α5β1 in endothelial cells (27). Furthermore, contacts between cultured keratinocytes have been shown to be disrupted by antibodies directed against α3β1 and β1 (6). Mediation of cellular cohesion by integrins could result from direct binding of integrins to counter-receptors present on adjacent cells or by neighboring cells jointly binding to extracellular matrix proteins deposited in the intercellular space.

The results of this study demonstrate the existence of a previously unrecognized member of the integrin family that is widely expressed in vivo in differentiated cells that are not actively involved in migration, proliferation, or heterotypic interactions with other cells. These data suggest that this integrin may function in some aspect of normal tissue cohesion or homeostasis. More definitive functional characterization will require identification of the ligand or ligands for this receptor and the development of reagents that specifically interfere with its function.

We thank Elizabeth Wayner (University of Minnesota, Minneapolis, MN) for providing the mAb P5D2, Ann Weinacker and Aileen Chen for developing the mAb R6G9, David Erle and Lynn Schnapp for helpful discussions during the course of this work, and Angela Wang for her assistance with establishing our immunohistochemistry protocol.

This work was supported by grants HL/AI33259, HL47412, CA53259, and HL191551 from the National Institutes of Health, and grant RT338 from the University of California Tobacco-Related Disease Research Program. C. Rüegg was supported by a fellowship from the American Heart Association, California Affiliate.

Received for publication 21 June 1993 and in revised form 27 August 1993.

References

1. Albelda, S. M. 1991. Endothelial and epithelial cell adhesion molecules. *Am. J. Respir. Cell Mol. Biol.* 4:195–203.
2. Angraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.
3. Arnaout, M. A., O. E. Remold, M. W. Pierce, P. Harris, and D. G. Tenen.

1988. Molecular cloning of the alpha subunit of human and guinea pig leukocyte adhesion glycoprotein Mo1: chromosomal localization and homology to the alpha subunits of integrins. *Proc. Natl. Acad. Sci. USA.* 85:2776–2780.

4. Bossy, B., W. E. Bossy, and L. F. Reichardt. 1991. Characterization of the integrin alpha 8 subunit: a new integrin beta 1-associated subunit, which is prominently expressed on axons and on cells in contact with basal laminae in chick embryos. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2375–2385.
5. Briesewitz, R., M. R. Epstein, and E. E. Marcantonio. 1993. Expression of native and truncated forms of the human integrin alpha 1 subunit. *J. Biol. Chem.* 268:2989–2996.
6. Carter, W. G., E. A. Wayner, T. S. Bouchard, and P. Kaur. 1990. The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. *J. Cell Biol.* 110: 1387–1404.
7. Cerf-Bensussan, N., B. Begue, J. Gagnon, and T. Meo. 1992. The human intraepithelial lymphocyte marker HML-1 is an integrin consisting of a beta 7 subunit associated with a distinctive alpha chain. *Eur. J. Immunol.* 22:273–277.
8. Cerf-Bensussan, N., A. Jarry, N. Brouse, B. Lisowska-Grospierre, D. Guy-Grand, and C. Griscelli. 1987. A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. *Eur. J. Immunol.* 17:1279–1285.
9. Corbi, A. L., T. K. Kishimoto, L. J. Miller, and T. A. Springer. 1988. The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit. Cloning, primary structure, and relation to the integrins, von Willebrand factor and factor B. *J. Biol. Chem.* 263:12403–12411.
10. Corbi, A. L., L. J. Miller, K. O'Connor, R. S. Larson, and T. A. Springer. 1987. cDNA cloning and complete primary structure of the alpha subunit of a leukocyte adhesion glycoprotein, p150,95. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4023–4028.
11. Dittel, B. N., J. B. McCarthy, E. A. Wayner, and T. W. LeBien. 1993. Regulation of human B-cell precursor adhesion to bone marrow stromal cells by cytokines that exert opposing effects on the expression of vascular cell adhesion molecule-1 (VCAM-1). *Blood.* 81:2272–2282.
12. Erle, D. J., C. Ruegg, D. Sheppard, and R. Pytela. 1991. Complete amino acid sequence of an integrin beta subunit (beta 7) identified in leukocytes. *J. Biol. Chem.* 266:11009–11016.
13. Erle, D. J., D. Sheppard, J. Breuss, C. Ruegg, and R. Pytela. 1991. Novel integrin alpha and beta subunit cDNAs identified in airway epithelial cells and lung leukocytes using the polymerase chain reaction. *Am. J. Respir. Cell Mol. Biol.* 5:170–177.
14. Fitzgerald, L. A., B. Steiner, S. C. J. Rall, S. S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin." *J. Biol. Chem.* 262:3936–3939.
15. Fritz, J. D., M. L. Greaser, and J. A. Wolff. 1991. A novel 3' extension technique using random primers in RNA-PCR. *Nucleic Acids Res.* 19:3747.
16. Gresham, H. D., J. L. Goodwin, P. M. Allen, D. C. Anderson, and E. J. Brown. 1989. A novel member of the integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. *J. Cell Biol.* 108: 1935–1943.
17. Harlow, E., and D. Lane. 1988. Immunoaffinity purification. *In* Antibodies: A Laboratory Manual. E. Harlow and D. Lane, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 521–523.
18. Hemler, M. E. 1991. *In* Receptors for Extracellular Matrix Proteins. R. P. Mecham and J. A. McDonald, editors. Academic Press, San Diego. 255–299.
19. Hemler, M. E., C. Huang, and L. Schwartz. 1987. The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. *J. Biol. Chem.* 262:3300–3309.
20. Hogervorst, F., I. Kuikman, A. E. von dem Borne, and A. Sonnenberg. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:765–770.
21. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11–25.
22. Hynes, R. O., E. E. Marcantonio, M. A. Stepp, L. A. Urry, and G. H. Yee. 1989. Integrin heterodimer and receptor complexity in avian and mammalian cells. *J. Cell Biol.* 109:409–420.
23. Ignatius, M. J., T. H. Large, M. Houde, J. W. Tawil, A. Barton, F. Esch, S. Carbonetto, and L. F. Reichardt. 1990. Molecular cloning of the rat integrin alpha 1-subunit: a receptor for laminin and collagen. *J. Cell Biol.* 111:709–720.
24. Kaufmann, R., D. Frosch, C. Westphal, L. Weber, and C. E. Klein. 1989. Integrin VLA-3: ultrastructural localization at cell-cell contact sites of human cell cultures. *J. Cell Biol.* 1807–1815.
25. Kilshaw, P. J., and S. J. Murant. 1990. A new surface antigen on intraepithelial lymphocytes in the intestine. *Eur. J. Immunol.* 20: 2201–2207.
26. Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1986. Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell.* 48:681–690.
27. Lampugnani, M. G., M. Resnati, E. Dejana, and P. C. Marchisio. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. *J. Cell Biol.* 112:479–490.
28. Larson, R. S., A. L. Corbi, L. Berman, and T. Springer. 1989. Primary structure of the leukocyte function-associated molecule-1 alpha subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* 108:703–712.
29. Laudet, V., C. Hanni, J. Coll, F. Catzeflis, and D. Stehelin. 1992. Evolution of the nuclear receptor gene superfamily. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1003–1013.
30. Law, S. K., J. Gagnon, J. E. Hildreth, C. E. Well, C. E. Willis, and A. J. Wong. 1987. The primary structure of the beta-subunit of the cell surface adhesion glycoproteins LFA-1, CR3, and p150,95 and its relationship to the fibronectin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:915–919.
31. Massia, S. P., and J. A. Hubbell. 1992. Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is mediated by integrin alpha 4 beta 1. *J. Biol. Chem.* 267:14019–14026.
32. Moyle, M., M. A. Napier, and J. W. McLean. 1991. Cloning and expression of a divergent integrin subunit beta 8. *J. Biol. Chem.* 266:19650–19658.
33. Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.
34. Ramaswamy, H., and M. E. Hemler. 1990. Cloning, primary structure and properties of a novel human integrin beta subunit. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1561–1568.
35. Rojiani, M. V., B. Finlay, V. Gray, and S. Dedhar. 1991. In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. *Biochemistry.* 30:9859–9866.
36. Rosen, G. D., J. R. Sanes, R. LaChance, J. M. Cunningham, J. Roman, and D. C. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. *Cell.* 69:1107–1119.
37. Sheppard, D., C. Rozzo, L. Starr, V. Quaranta, D. J. Erle, and R. Pytela. 1990. Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.* 265:11502–11507.
38. Song, W. K., W. Wang, R. F. Foster, D. A. Bielser, and S. J. Kaufman. 1992. H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.* 117:643–657.
39. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino. M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor alpha subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.
40. Suzuki, S., J. Z. Huang, and H. Tanihara. 1990. Cloning of an integrin beta subunit exhibiting high homology with integrin beta 3 subunit. *Proc. Natl. Acad. Sci. USA.* 87:5354–5358.
41. Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:757–763.
42. Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the alpha 4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1361–1368.
43. Takada, Y., and M. E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell. Biol.* 109:397–407.
44. Takada, Y., E. Murphy, P. Pil, C. Chen, M. H. Ginsberg, and M. E. Hemler. 1990. Molecular cloning and expression of the cDNA for alpha 3 subunit of human alpha 3 beta 1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. *J. Cell Biol.* 115:257–266.
45. Tamura, R. N., C. Rozzo, L. Starr, J. Chambers, L. F. Reichardt, H. M. Cooper, and V. Quaranta. 1990. Epithelial integrin alpha 6 beta 4: complete primary structure of alpha 6 and variant forms of beta 4. *J. Cell Biol.* 111:1593–1604.
46. Tsuji, T., F. Yamamoto, Y. Miura, K. Takio, K. Titani, S. Pawar, T. Osawa, and S. Hakomori. 1990. Characterization through cDNA cloning of galactoprotein b3 (Gap b3), a cell surface membrane glycoprotein showing enhanced expression on oncogenic transformation. Identification of Gap b3 as a member of the integrin superfamily. *J. Biol. Chem.* 265:7016–7021.
47. Yuan, Q., W. M. Jiang, D. Hollander, E. Leung, J. D. Watson, and G. W. Krissansen. 1991. Identity between the novel integrin beta 7 subunit and an antigen found highly expressed on intraepithelial lymphocytes in the small intestine. *Biochem. Biophys. Res. Commun.* 176:1443–1449.

Immunity, Vol. 3, 683–690, December, 1995, Copyright © 1995 by Cell Press

A Novel Leukointegrin, αdβ2, Binds Preferentially to ICAM-3

Monica Van der Vieren,* Hai Le Trong,* Christi L. Wood,* Peter F. Moore,† Tom St. John,* Donald E. Staunton,* and W. Michael Gallatin*
*ICOS Corporation
22021 Twentieth Avenue Southeast
Bothell, Washington 98021
†Department of Pathology, Microbiology and Immunology
School of Veterinary Medicine
University of California at Davis
Davis, California 95616

Summary

The leukocyte-restricted β2 (CD18) integrins mediate cell adhesion in a variety of events essential for normal immune function. Despite extensive research in this field, only three members of this integrin subfamily have been described: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150,95). We have identified a cDNA encoding a fourth α chain, αd, that associates with CD18. The αd subunit is more closely related to CD11b and CD11c than to CD11a. This integrin is expressed at moderate levels on myelomonocytic cell lines and subsets of peripheral blood leukocytes, and more strongly on tissue-compartmentalized cells such as foam cells, specialized macrophages found in aortic fatty streaks that may develop into atherosclerotic lesions. The αd/CD18 molecule exhibits preferential recognition of ICAM-3 over ICAM-1.

Introduction

The integrins are a family of heterodimeric membrane-bound glycoproteins that mediate homotypic and heterotypic cell–cell adhesion in a broad range of biological contexts (Hynes, 1992; Larson and Springer, 1990). A subfamily of integrins expressed in leukocytes is defined by a common beta chain, β2 (CD18), that pairs with a distinct subgroup of α chains (Hynes, 1987). These α chains contain a region of approximately 200 aa residues, designated the I (inserted) or A domain, that shares structural homology to ligand-binding domains in Von Willebrand factor and other proteins (Larson and Springer, 1990; Michishita et al., 1993; Lee et al., 1995) and has recently been implicated in ligand recognition (Landis et al., 1993; Diamond et al., 1993; Randi and Hogg, 1994; Zhou et al., 1994). The leukointegrins are critical to immune function: absence of CD18 cell surface expression in patients with leukocyte adhesion deficiency (LAD) results in impairment of a variety of immune functions, including neutrophil transendothelial migration, macrophage oxidative burst and phagocytosis, and lymphocyte proliferation (Springer et al., 1984; Anderson and Springer, 1987).

To date, the leukointegrin subfamily includes CD11a/CD18, CD11b/CD18, and CD11c/CD18, also known as LFA-1, Mac-1, and p150,95, respectively (Kishimoto et al., 1987; Corbi et al., 1987, 1988; Larson et al., 1989). LFA-1 cell surface expression is constitutive on a broad range of leukocytes, while expression of Mac-1 and p150,95 is more restricted and inducible by inflammatory mediators (Larson and Springer, 1990). LFA-1 and Mac-1 mediate cell–cell contact by interacting with the intercellular adhesion molecules (ICAMs), immunoglobulin superfamily members (Hynes, 1992; Larson and Springer, 1990). Whereas both LFA-1 and Mac-1 bind to ICAM-1, only LFA-1 has been demonstrated to bind to ICAM-3. ICAM-3 is highly expressed on resting lymphocytes and its linkage to intracellular signal transduction pathways contributes to lymphocyte activation (Vives, 1994). Here, we report the identification of a novel fourth leukointegrin α chain, αd, that contains an I or A domain and is most homologous to Mac-1 and p150,95. The αd protein is expressed on myelomonocytic cell lines in a pattern distinct from that of CD11b and CD11c. This molecule is expressed predominantly in splenic red pulp on macrophages and granulocytic cells in an overlapping but nonidentical pattern with the other leukointegrin α chains. The protein can be detected at low levels relative to CD11a and CD11b on subsets of peripheral blood cells. In addition, it is present on lipid laden macrophages in aortic fatty streaks. This α chain forms a heterodimer with β2 that binds ICAM-3 but not ICAM-1 when expressed in Chinese hamster ovary (CHO) cells.

Results and Discussion

For analyses of β2 integrin function in the dog, Danilenko et al. (1992) generated a panel of monoclonal antibodies (MAbs) against canine CD18 complexes. Interestingly, one of these canine-specific antibodies recognized a CD18-associated protein with histologic distribution and apparent molecular weight distinct from that of the known leukointegrins (Danilenko et al., 1995). The expression pattern of this molecule was restricted mainly to tissues and differed from the distribution of CD11b and CD11c. Amino-terminal sequence derived from this α chain indicated leukointegrin homology; however, this protein could not be defined as the distinct product of a novel gene since no sequence information regarding canine CD11a, CD11b, or CD11c was available for comparison. A priori, these findings might also be explained by cell-type specific mRNA splicing, posttranslational modification, or unexpected pairing of CD18 with known integrin α chains from other subfamilies.

We presumed that DNA fragments amplified from canine cDNA could be used in cross-species hybridizations to identify a human homolog, thus permitting direct comparisons with known α subunits. We obtained peptide sequences for polymerase chain reaction (PCR) primer design from fragmentation of the αd protein isolated from

canine spleen. Degenerate oligonucleotides based on 2 of 9 peptides sequenced were used as primers in a PCR with canine splenic cDNA to amplify a 1 kb DNA fragment. This fragment was used as a probe to identify a homologous clone (19A2) in a human spleen cDNA library under stringent hybridization conditions. Although no exact matches with any sequence in the National Center for Biotechnology Information databases were found, sequence homologies to the known leukointegrin α chains suggested that the protein encoded by clone 19A2 belonged to this integrin subfamily. This clone appears to share sequence homology with a partial genomic clone identified independently (D. Wong, personal communication). We have designated this previously unidentified α chain αd.

As shown in Figure 1, clone 19A2 encompasses the entire coding region for the mature protein, including 13 residues of leader sequence. Two additional clones have been isolated and used to confirm this sequence and to indicate that a methionine precedes the 19A2 leader sequence. The putative amino acid sequence shares greater identity to CD11b (60%) and CD11c (66%) than to CD11a (36%). The molecular mass of the mature core protein is predicted to be 125 kDa with an extracellular domain encompassing 1084 residues followed by a hydrophobic 23-residue potential transmembrane domain and a 38-residue cytoplasmic tail. There are eleven potential N-linked glycosylation sites. The seven homologous internal repeats observed in CD11a, CD11b, and CD11c are also conserved in αd.

Also present in αd is a 204-residue segment homologous to the I or A domain recently implicated in ligand recognition (Landis et al., 1993; Diamond et al., 1993; Randi and Hogg, 1994; Edwards et al., 1995). The I domain

```
                    I
mtvllsvlss yhqFNLDVEE PTIFQEDAGG PGQSVVQPGG SRLVVGAPLE VVAANQTGR 60
                   II
LYDCAAATGN QPIPLHIRPE AVEMSLGLTL AASTNGSRLL ACGPTLHRVC GENSYSKGS 120
CLLLGSRMII QTVPDATPEC PHQEMGIVFL IDGSGSIDQN DFNMMKEFVQ AVMGQFEGT 180
DTLFALMQYS NLKTHFTFTD FRTSPSQQSL VDPIVQLKGL TPTATGILTV VTQLPHSKN 240
GARESAKKLL VITDGQKYKD PLEYSDVIPQ AEKAGIIRYA IGVGHAFQGR TAREFLNTI 300
                                                  III
SSAPEQGNVF VDNFAALGSI QKQLQEKIYA VEGTQSRASS SFQHEMSQEG FSTALTMDG 360
                  IV
LFLGAVGSFS WSQAFLYPPN MSPTFINMSQ ENVDMRDSYL GYSTELALWK GVQNLVLGA 420
                 V
PRYQNTGKAV IFTQVSRQWR KAEVTGTQIG SYFGASLCSV DVDSDGSTDL ILIGAPHYY 480
                      VI
EQTRGGQVSV CPLPRGQRVQW QCDAVLRGEQ GNPWGRFGAA LTVLGDVNED KLIDVAIGA 540
                        VII
PQEQENRGAV YLFHGASESGI SPSHSQRIAS SQLSPRLQYF GQALSGGQDL TQDGLMDLA 600
VGARGQVLLL RSLPVLKVGVA MRFSPVEVAK AVYRCWEEKP SALRAGDATV CLTIQKSSL 660
DQLGDIQSSVR FDLALDPGRL TSRAIFNETK NPTLTRRKTL GLGIMCETLK LLLPDCVED 720
VVSPIILNLN  SLVREPIPSP QNLRPVLAVG SQDLFTASLP FEKNCGQDGL CEGDLGVTL 780
SFSGLQTLTVQ SSLELNVIVT VNNAGEDSYG TVVSLYYPAG LSHRRVSGAQ KQPHQSALR 840
LACETVPTEDS GLRSSRCSVN HPIPHEGSNG TFIVTFDVSY KATLGDRKLM RASASEDN 900
KASSSKATFQL ELPVKYAVYT MISRQEESTK YFNFATSDEK NGKEAEHRYR VNNLSQRDL 960
AISINFWVPVL LNGVAVWDVV KEAPSQSLPC VSERKPPQHS DFLTQISRSP MLDCSIADC 1020
LQFRCDVPSFS VQEELDFTLK GNLSFGWVRE TLQKEVLVVS VAEITFDTSV YSQLPQQEA 1080
FNRAQNENVLE EDEVYNAIPI IMGSSVGALL LLALITATLY KSGFFKRHYK EMLEDKPED 1140
                  ********************
TATFSGDDFSC VAPHVPLS                                              1159
```

Figure 1. Amino Acid Sequence of the αd Subunit

Leader sequence is denoted by lowercase letters. Additional cDNA clones have confirmed the single methionine preceding the leader sequence from clone 19A2. Homologous internal repeats are labeled with roman numerals at their amino-terminal ends. The I domain is underlined. Putative cation binding sites are indicated in bold letters; the MIDAS motif is included in the first cation binding site. Potential N-linked glycosylation sites are underlined. The transmembrane domain, denoted by carets, immediately precedes the cytoplasmic domain. The cytoplasmic KLGFFKR motif is in italics. The nucleotide sequence of clone 19A2 can be obtained from the Genbank database (accession number U37028).

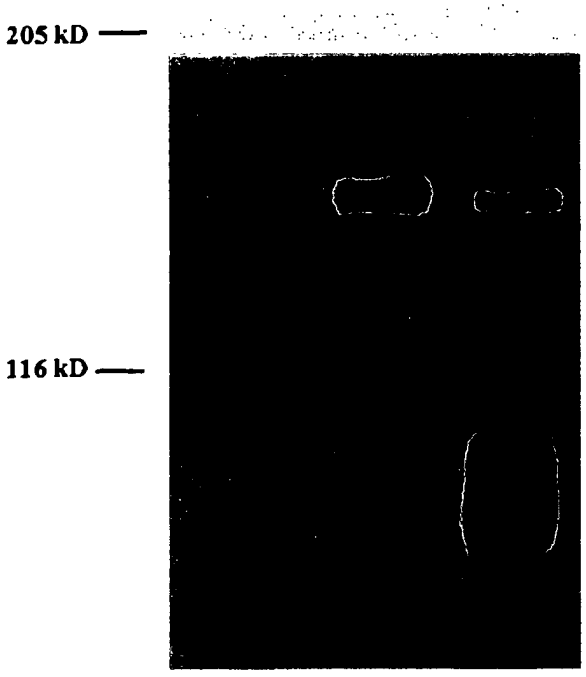


Figure 2. αd Forms a Heterodimer with β2, CD18

Heterodimeric complexes immunoprecipitated from lysates of biotinylated αd/CD18 CHO transfectants with either αd- or CD18-specific MAb were visualized with peroxidase-conjugated strepavidin. Lane 1, αd/CD18 CHO plus mouse IgG; lane 2, αd/CD18 CHO plus TS1/18; lane 3, αd/CD18 CHO plus 169A. Cell surface expression of CD18 required cotransfection of the αd cDNA: transient transfections of the β chain construct alone did not result in detectable CD18 expression by flow cytometry and in immunoprecipitation experiments (data not shown).

amino acid sequence is 36%, 62%, and 57% identical to the same regions in CD11a, CD11b, and CD11c, respectively. αd also contains identical residues defined in the I domain of CD11b as a metal ion-dependent adhesion site (MIDAS) necessary for ligand binding (Michishita et al., 1993, Lee et al., 1995), as well as three additional cation binding sites conserved in leukointegrin α subunits. There is similarity between the peptide sequences from CD11b, which have been demonstrated to interact with complement component iC3b (Ueda, et al., 1994) and the homologous region in αd. The moderate level of sequence conservation in these regions suggests that while αd may share ligands with the known leukointegrins, it may also recognize distinct cell surface or extracellular matrix proteins.

The cytoplasmic domains of integrin α subunits are implicated in integrin activation. These domains are typically unrelated to one another, but conserved across species. The putative cytoplasmic sequence of αd differs markedly from that of CD11a, CD11b, and CD11c (10%, 16%, and 18% identity, respectively), except for the membrane proximal KXGFFKR motif, conserved among all α integrins, that is involved in affinity regulation from the cytoplasmic side, or inside-out signaling (Ginsberg et al., 1992, Rojiani et al., 1991). Engagement of αd, even in a similar cellular context as other leukointegrins, may involve interactions with a distinct subset of cytosolic proteins and may have functional consequences specific to αd.

Cotransfection of the cDNAs encoding αd and CD18 into CHO cells resulted in cell surface expression of a heterodimeric molecule, both chains of which were immunoprecipitated by either anti-CD18 or anti-αd MAb (Figure 2).

The αd-specific MAb used in this experiment was generated from mice injected with CD18 complexes isolated from human splenic lysates precleared of CD11a and CD11b. Supernatants from resulting immunoglobulin G (IgG)-secreting hybridomas were screened by flow cytometry for reactivity with αd/CD18 CHO transfectants but not with JY cells, which had been previously determined by Northern blot analysis to be αd negative. The IgG1 antibody secreted by clone 169A was determined to be specifically reactive with αd only by flow cytometric analysis of stable αd/CD18, CD11a/CD18, CD11b/CD18, and CD11c/CD18 CHO transfectants (Table 1, stable transfectants). This antibody was not observed to have inhibitory activity in the functional assays presented here.

To ascertain cell-type expression of αd and its relationship to expression of other leukointegrins, the presence of αd protein on human lymphoid and myelomonocytic cell lines and on freshly isolated peripheral blood leukocytes (PBLs) was evaluated by flow cytometry using the MAb 169A and compared with expression of CD11a, CD11b, and CD11c (Table 1). αd expression is modulated with differentiation of the myeloid cell lines HL60 and THP-1 (Table 1, cell lines). DMSO treatment, which induces granulocyte differentiation in the promyelomonocytic line HL60 (Collins, 1987) did not affect the percent of cells positive for αd, which was expressed on more cells than CD11c

Table 1. Flow Cytometric Analysis of αd Surface Expression on Cell Lines, PBL, and Transfectants

Cell lines	αd	CD11a	CD11b	CD11c	PBL	αd	CD11a	CD11b	CD11c	Stable Transfectants	αd	CD11a	CD11b	CD11c
Myeloid					Lymphocytes	25 (83)	120 (93)	67 (45)	27 (23)	αd/CD18 CHO	64 (95)	19 (1)	11 (1)	9 (1)
HL60										LFA-1 CHO	10 (1)	45 (70)	8 (1)	12 (1)
No stimulation	36 (82)	102 (98)	19 (38)	13 (3)	Monocytes	51 (98)	132 (99)	418 (90)	84 (99)					
PMA	27 (42)	45 (78)	54 (71)	31 (72)										
DMSO	20 (87)	74 (78)	18 (87)	28 (15)										
THP-1					Granulocytes									
No stimulation	42 (66)	55 (72)	81 (52)	82 (35)	No stimulation	19 (94)	46 (99)	330 (99)	23 (98)					
PMA	59 (28)	55 (35)	170 (99)	101 (95)	PMA	54 (95)	28 (95)	500 (96)	31 (95)					
					fMLP	70 (92)	71 (91)	403 (98)	62 (97)					
Lymphoid														
Jurkat (T cell)	7 (7)	16 (88)	9 (42)	8 (4)										
Ramos (B cell)	5 (5)	9 (84)	5 (11)	5 (5)										
JY (B cell)	11 (13)	17 (86)	12 (19)	17 (8)										
KU812 (mast)	11 (16)	14 (40)	19 (47)	4 (30)										

MAbs were used to detect surface expression of αd, CD11a, CD11b, or CD11c, respectively, on cell lines, peripheral blood leukocytes, and stable CHO transfectants. Data shown are from a single experiment representative of 3–5 separate experiments. Gates were set to exclude isotype-matched irrelevant controls with a background of 5% positive cells. Results are expressed as a mean fluorescence intensity of 10^2 positive cells per determination, with percent positive in parentheses.

both before and after treatment. However, phorbol myristate acetate (PMA) stimulation of both HL60 and THP-1 cells along a macrophage-like differentiation pathway resulted in increased CD11b and CD11c expression levels, with a corresponding reduction in percent positive for αd. The majority of peripheral blood lymphocytes express low levels of αd relative to CD11a and CD11b (Table 1, PBL); these patterns do not vary significantly among donors. Granulocytes were also observed to express αd, although at significantly lower levels than CD11b. Expression appeared to increase rapidly on granulocytes with PMA or f-Met Leu-Phe (fMLP) stimulation, consistent with recruitment of an intracellular pool of αd in these cells. Thus, levels of αd expression may differ from that of CD11a, CD11b, or CD11c depending on the cell type and activation or differentiation state.

Comparative expression of the previously identified leukointegrin α chains and αd was assessed by immunohistology in the spleen because of the highly ordered structure of the lymphoid domains and the presence of diverse cell types. The overall pattern of αd distribution in the red pulp of spleen was overlapping with but not identical to that of CD11a, CD11b, and CD11c (Figure 3). Whereas CD11a was diffusely expressed by leukocytes in all splenic domains, including lymphoid domains of the white pulp and marginal zone and cords and sinuses of the red pulp, CD11b, CD11c, and αd expression patterns were more restricted. CD11b staining was prominent on granulocytes in the red pulp cords and sinuses. CD11c was expressed by dendritic cells in the periarterial lymphoid sheaths, marginal zones, and follicles of the white pulp. The distribution of αd was predominantly localized to the red pulp cords and sinuses on small and large mononuclear cells and granulocytes. Expression of αd in the white pulp was limited to scattered dendritic cells occurring less frequently than $CD11c^+$ cells. Although relative intensity of splenic integrin staining appeared to vary among three tissue donors (data not shown), probably owing to differences in age, health, or mortality factors, the distribution patterns did not vary.

A preliminary evaluation of αd expression in pathologic tissue with macrophage-specific etiology was performed using sections of abdominal aorta selected for the incidence of fatty streaks, which histologically consisted of subintimal infiltrates of smaller leukocytes and aggregates of foamy macrophages (Pathobiological Determinants of Atherosclerosis in Youth [PDAY] Research Group). These highly specialized macrophages are implicated in the establishment of subintimal aortic fatty streaks, which may progress to fulminant atherosclerotic lesions (Ross, 1993). Foamy macrophages can be identified in fatty streaks by detection of ingested lipid with the Oil Red O stain and differentiated from lipid-containing smooth muscle cells by specific markers. In the Oil Red O–positive sections we examined, a subset of the large lipid-laden cells reactive with an antibody to CD68, a macrophage-specific marker, expressed moderate levels of αd (Figure 3). These cells were not stained by an antibody to smooth muscle actin, confirming that the $αd^+$ foam cells were not of smooth muscle origin (data not shown). In addition, these macrophage-like cells did not stain with CD3, CD8, or CD20 antibodies (data not shown). Double-labeled aortic sections confirm a significant overlap of CD68 and αd staining. The αd-specific antibody did not react with aortic sections that were Oil Red O negative (data not shown).

Typically, the leukocyte integrins promote cell–cell contact by binding to the intercellular adhesion molecules (ICAMs) (Hynes, 1992; Larson and Springer, 1990). Soluble forms of ICAM-1 and ICAM-3 have been observed in serum with increased levels in certain pathologic conditions (Martin et al., 1995). Therefore, the ability of CHO cells expressing αd/CD18 or LFA-1 to bind soluble ICAM-3/Ig, ICAM-1/Ig, and VCAM-1/Ig chimeric proteins was assessed by flow cytometry (Figure 4A). The αd/CD18, LFA-1, and VLA-4 transfectants used in subsequent binding experiments were 60%–90% CD18 or α4 positive, with equivalent cell surface expression in individual experiments. In replicate experiments, ICAM-3/Ig, but not ICAM-1/Ig or VCAM-1/Ig, bound to αd/CD18 transfectants. The CD18 dependence of this binding was confirmed by its sensitivity to treatment with anti-CD18 antibody. Similar results were obtained for αd/CD18 cotransfectants in adhesion assays using immobilized ICAM-1/Ig or ICAM-3/Ig chimeras (Figure 4B). The αd/CD18 transfectants bound 2- to 4-fold greater to ICAM-3/Ig than to the bovine serum albumin (BSA) control, with no significant binding to ICAM-1/Ig.

Treatment of αd/CD18 transfectants with PMA or manganese, positive regulators of LFA-1 activation, did not significantly affect binding to CAMs in either of the adhesion assays described. CD11a/CD18 transfectants exhibited a PMA-dependent 3- to 5-fold increase in binding to ICAM-1 (Figure 4B), consistent with the activation requirement and apparent avidity reported for LFA-1/ICAM-1 interactions in this cellular context (Dustin and Springer, 1989; van Kooyk et al., 1989). Differences in αd and CD11a regulation may reflect unique cytosolic interactions mediated by their nonhomologous cytoplasmic tails.

In summary, we have described a previously unknown human leukointegrin α subunit that forms a functional heterodimer with CD18. Although structurally similar to the known α chains, divergence in key regions of this protein implies that αd/CD18 plays a different role in immune responses. For example, the distinct sequences of the αd I domain and cytoplasmic tail region suggest the potential for unique ligands, or unique binding sites on shared ligands, and affinity regulation specific to this integrin.

The predominant expression of αd observed on specialized cells in tissues suggests that the major functions of αd may be restricted to particular microenvironments. For example, the presence of αd on splenic red pulp macrophages may indicate a role for this integrin in phagocytosis of effete erythrocytes, bloodborne pathogens, and particulate matter from the blood. The involvement of foam cells in establishment of vascular lesions in certain types of atherosclerosis (Ross, 1993), combined with the observation that αd is expressed on those cells in fatty streaks, suggest that αd may contribute to macrophage-specific

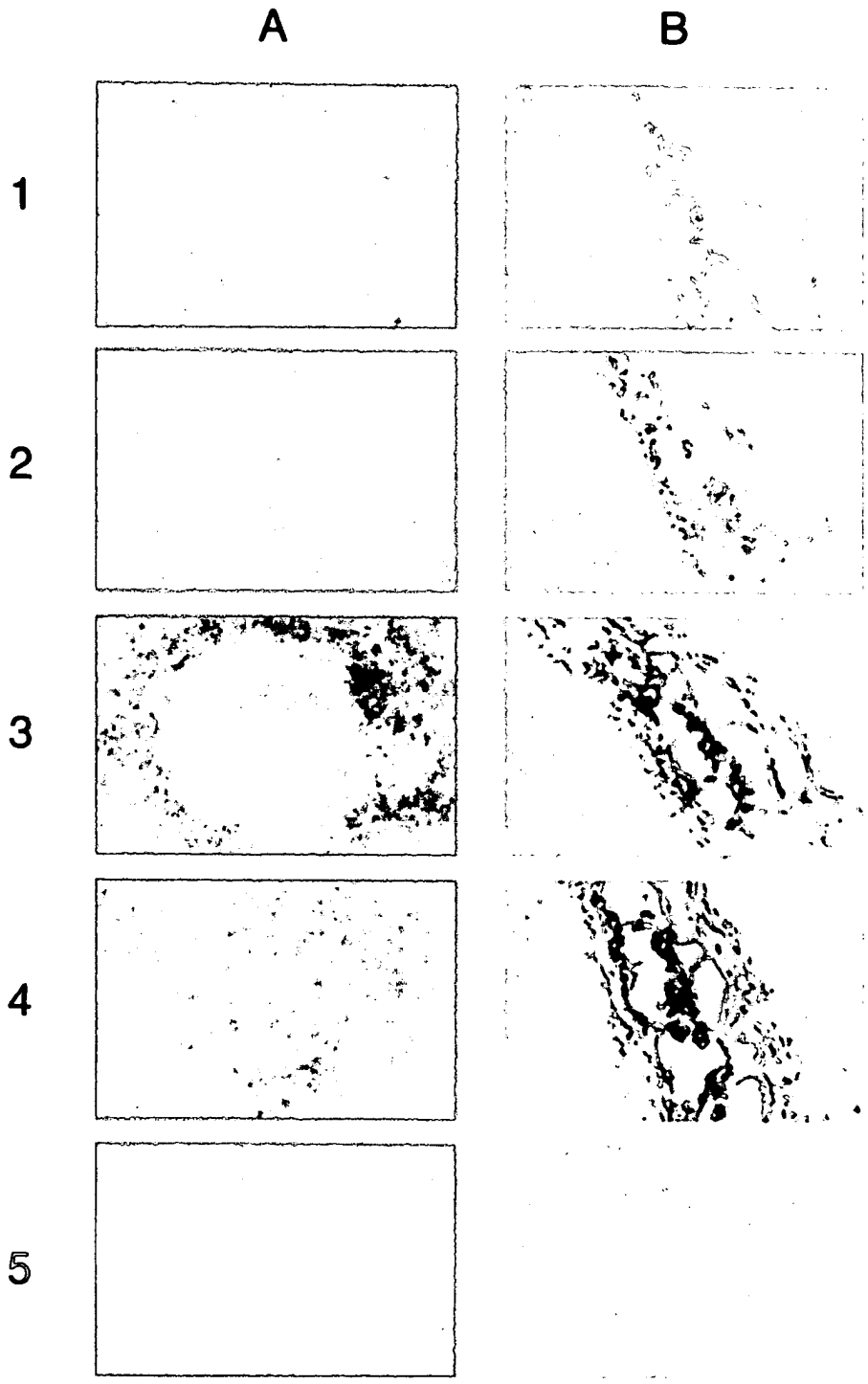
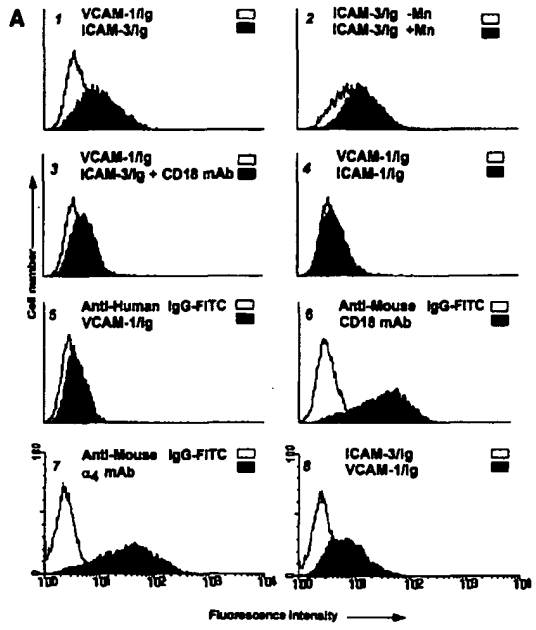
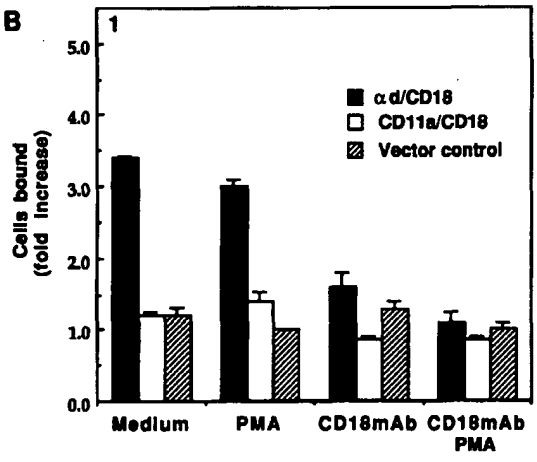


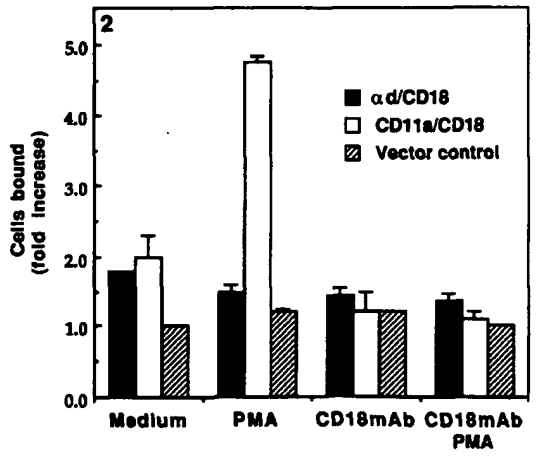
Figure 3. Expression of αd in Human Spleen and Aorta

(A) Fresh frozen splenic sections were acetone fixed and treated with antibodies against (1) αd, (2) CD11a, (3) CD11b, (4) CD11c, or (5) irrelevant mouse IgG1.

(B) Abdominal aortic sections were acetone fixed and treated with (1) Oil Red O, (2) MAb 169A (αd) (blue), (3) MAb EBM11 (CD68) (blue), (4) both 169A (blue) and EBM11 (brown), and (5) irrelevant mouse IgG1.







activities such as phagocytosis of modified lipoproteins in these lesions. Therefore, animal models of atherosclerosis may present one interesting venue to explore the in vivo function of αd.

The relevance of the low levels of αd observed on granulocytes, peripheral blood T cells, and monocytes may become evident when the full range of αd functions is defined. The presence of αd protein in CD18 immunoprecipitations from PBL may easily have been overlooked in the past due to overwhelming levels of other leukointegrins and similarity in apparent molecular weights of the α chains. It is possible that ligand specificity, activation regulation, and increased expression levels in particular environments may confer to αd different functions than other leukointegrins expressed by these cells.

The cell type and tissue distribution of human and canine αd protein expression is only partially conserved. As in the dog, αd is expressed on splenic red pulp macrophages (Danilenko et al., 1995) but not on Kupffer cells, resident macrophages of the liver (data not illustrated). However, the presence of αd on peripheral blood and splenic granulocytes from humans contrasts with the dog. This cross-species variation is perhaps unsurprising considering the presence of CD4 on canine, but not human granulocytes (Moore, et al., 1992). Other tissues that differ in αd distribution or intensity or both between the human and other species may be attributed either to species differences or to the variable characteristics of human donor tissues.

The capacity of αd/CD18 to bind both soluble and immobilized ICAM-3 implies a higher affinity interaction than that observed for LFA-1, which does not bind ICAM-3 in

Figure 4. αd/CD18 Binds to Soluble and Immobilized ICAM-3

The ability of stable αd/CD18 CHO transfectants to recognize soluble (4A) and immobilized (4B) ICAM-3 and ICAM-1 was determined.

(A) Transfectants were incubated in buffer with ICAM-3/Ig, ICAM-1/Ig, or VCAM-1/Ig. Bound protein was detected with FITC-conjugated anti-human immunoglobulin and subsequent flow cytometric analysis. (1) Comparison of ICAM-3/Ig and VCAM-1/Ig binding; (2) relative binding of ICAM-3/Ig in the presence or absence of manganese; (3) inhibition of ICAM-3/Ig binding by TS1/18 relative to VCAM-1/Ig binding; (4) comparison of ICAM-1/Ig and VCAM-1/Ig binding; (5) VCAM-1/Ig binding to transfectants relative to anti-human immunoglobulin tracer antibody only; (6) surface CD18 expression on αd/CD18 CHO transfectants, detected with the anti-CD18 MAb TS1/18; (7) α4 expression on VLA-4 CHO transfectants, detected with the antibody A4.1; and (8) binding of VCAM-1/Ig and ICAM-3/Ig to VLA-4 CHO transfectants. Mock-transfected and untransfected cells were not recognized by any fusion protein or by the FITC-conjugated tracer antibody (data not shown).

(B) CHO transfectants labeled intracellularly with calcein were incubated on microtiter plates coated with ICAM-3/Ig or ICAM-1/Ig. Fluorescence before and after washing was determined. Data is presented as fold increase of binding to ICAM/Ig over BSA control. Maximal binding of αd/CD18 CHO to ICAM-3 was 30%–50% of cells added to plates; for LFA-1 (PMA+) and ICAM-1, 20%–30%; and 5%–10% for all transfectants to BSA. (1) Binding of CHO transfectants to ICAM-3/Ig; (2) binding of CHO transfectants to ICAM-1/Ig. The CD11a/CD18 transfectants did not show significant binding to ICAM-3, perhaps owing to modest surface expression of LFA-1. No binding of αd/CD18 or CD11a/CD18 transfectants to VCAM-1/Ig was observed (data not shown).

a similar cellular context. In addition, the failure of αd/CD18 to bind soluble or immobilized ICAM-1 or VCAM-1 suggests an exclusive ICAM-3 recognition by this integrin. However, the αd subunit, like other leukointegrin α chains, probably recognizes multiple ligands and may exhibit additional CAM binding specificities when expressed in other cellular contexts in vivo. We are currently attempting to define the unique functional range of the αd molecule. Moreover, reevaluation of many leukointegrin-dependent immune phenomena may now be warranted to determine the contribution of this previously unrecognized family member.

Experimental Procedures

Isolation of the αd cDNA

αd/CD18 complexes affinity purified from detergent lysates of canine spleen (Danilenko et al., 1992) were subjected to preparative SDS–PAGE in 7% polyacrylamide and transferred to PVDF membranes. The Coomassie-stained 150 kDa band was fragmented with cyanogen bromide (CNBr) in 70% formic acid. CNBr-cleaved protein was eluted once with 60% acetonitrile, 0.1% TFA, and once with 80% acetonitrile, 0.08% TFA. The CNBr fragments were dried by vacuum, reduced and alkylated, and digested with trypsin (Stone et al., 1992). Resulting peptides were separated on a Waters Associates high pressure liquid chromatography system using a Vydac C-18 column and sequenced by Edman degradation.

Degenerate oligonucleotides were designed on the peptide sequences (FNLDVEEPMVFQED) and (FQEGFSSVLT). Double-stranded cDNA was generated from canine splenic poly (A)$^+$ mRNA (Gubler and Hoffman, 1983). A PCR containing the oligonucleotides (RAANCCYTCYTGRAAACTYTC) and (TTYAAYYTNGAYGTNGARGARCC) and 200 ng cDNA yielded a 1 kb product, which was ligated into the pCRII vector (Invitrogen, San Diego, California) and sequenced by the Sanger dideoxy termination method.

Oligo dT-primed double-stranded cDNA was prepared from poly(A)$^+$ RNA isolated from normal human spleen. BstXI linkers were added to the cDNA prior to cloning into pCDNA.Amp (Invitrogen). The ^{32}P-labeled canine αd fragment was added to colony replicas in prehybridization buffer (5× SSC, 10× Denhardt's, 1% SDS, 30% formamide), and allowed to hybridize overnight at 42°C. The final stringency wash was at 65°C in 1× SSC, 0.1% SDS. A single clone, 19A2, was identified and sequenced on the sense strand by the nested deletions technique. The antisense strand was sequenced for confirmation.

Expression and Analysis of the αd cDNA

An oligonucleotide was designed to include a 5′ EcoRI site, modified Kozak consensus sequence (Kozak, 1987), an adenosine, and sequence overlapping the 5′ end of clone 19A2 (AGTTACGAATTCGCCACCATGACCTTC GGCACTGTG). A 706 bp fragment was amplified from clone 19A2 using this oligo and a specific internal oligonucleotide from the antisense strand (CCACTGTCAGGATGCCCGTG). The PCR product was restricted with EcoRI and BamHI and ligated to the 3.2 kb BamHI–XbaI fragment from 19A2. The resulting EcoRI–XbaI fragment was ligated into pcDNA3 (Invitrogen) resulting in the plasmid pATM.D12. The cDNAs encoding human CD11a, CD18, and CD29 (β1) were each subcloned into the expression vector pDC1. A pCDM8 construct containing the CD11c cDNA was obtained from F. Sanchez-Madrid (Universidad Autonoma de Madrid, Spain). The CD49d (α4) and CD11b encoding cDNAs were subcloned into the pcDNA.1 vector (Invitrogen). The plasmids pATM.D12 and pDC1.CD18 were used to transfect DHFR–DG44. CHO cells (obtained from L. Chasen, Columbia University, New York) by electroporation. The plasmid pDC1.CD11a was transfected along with pDC1.CD18 as a positive control. Vectors expressing α4 and β1 were cotransfected to provide a positive control for VCAM-1 binding experiments. Stably transfected clones were selected in nucleoside-deficient medium.

Biotinylated (Cole et al., 1987) CD18 complexes were precipitated from detergent lysates of CHO transfectants with the monoclonal antibody TS1/18 (American Type Culture Collection [ATCC] HB203) or 169A (anti-αd). Biotinylated protein was isolated under reducing conditions on 8% polyacrylamide gels and transferred to PVDF membranes. Biotinylated proteins were detected with Strepavidin POD (Boehringer Mannheim) diluted 1:6000 in TBS-T, 0.3% BSA. Hyperfilm (Amersham) was used to expose the membranes for 0.5–2 min after development with enhanced chemiluminescence reagents (Amersham).

Generation of αd-Specific MAbs

Detergent lysates were made as previously described (Danilenko et al., 1992) from 100 g of human spleen obtained from the University of Utah. Lysates were clarified by centrifugation and sequentially precleared of LFA-1 and Mac-1 with Affigel 10 resin to which the antibody TS1/22.1 (ATCC HB202) or 44aacb (ATCC HB249) was bound. Remaining CD18 complexes were affinity purified with the murine antibody TS1/18. BALB/c mice were immunized three times at 21 day intervals with 30 μg/mouse protein in adjuvant. The spleen from a single mouse was fused with NS-1 myeloma cells and hybridomas were obtained according to standard procedures. Culture supernatants were analyzed by flow cytometry for the ability to react with αd/CD18 CHO cells but not with JY cells, which in our hands express only CD11a/CD18. The clone 169A (IgG1) was isolated after two rounds of subcloning. Antibody purified from exhausted hybridoma supernatant on a PROSEP-A column (Bioprocessing Limited) according to package directions was used in the flow cytometric experiments presented; histologic analysis was performed using supernatants.

Flow Cytometric Analysis of αd Expression

Surface expression of leukointegrin α chains on cell lines, PBLs, and transfectants was detected with the murine MAbs 169A (αd), TS1/22 (CD11a, ATCC), 44aacb (CD11b, ATCC), and BU15 (CD11c, Dako). Human PBLs were isolated from blood on a Histopaque 1077 (Sigma) gradient using standard procedures. Granulocytes were isolated on Ficoll–Hypaque gradients (Ferrante and Thong, 1982). Cellular Fc were blocked with 1 mg/ml human IgG (Sigma) in RPMI with 2% fetal bovine serum and 0.01% sodium azide (FACS buffer) for 30 min on ice. Cells (10^6) per group were incubated on ice in 100 μl FACS buffer with primary antibody at 10 μg/ml or 100 μl hybridoma supernatant. Bound antibody was detected with sheep anti-mouse IgG–fluorescein isothiocyanate (FITC) (Sigma), followed by washing and analysis on a Becton-Dickinson FACscan using Lysis II software.

Immunohistology

Frozen sections from human abdominal aorta (PDAY Research Group) were stained with irrelevant murine IgG or MAb 169A (αd). Bound antibody was detected using rabbit anti-mouse IgG (Dako) and mouse APAAP reagent (Dako) with subsequent development with Substrate Kit III (Vector). Sections were counterstained with Nuclear Fast Red (Vector). Human spleen and liver sections (National Disease Research Interchange) were treated in the same fashion, but with the addition of MAbs against CD11a (TS1/22, ATCC), CD11b (44aacb, ATCC), and CD11c (BU15, Dako). Aortic sections stained with Oil Red O were dehydrated with 100% propylene glycol before staining. For double staining, MAb EBM11 (CD68)-treated sections were incubated successively with supersaturating concentrations of peroxidase-conjugated anti-mouse IgG (1:100, Dako) and peroxidase-conjugated swine anti-rabbit IgG (1:30, Dako). Sections were blocked with swine anti-rabbit IgG before addition of MAb 169A. Slides were developed with the DAB (3.3 diaminobenzidine) kit (Vector) and the AP (alkaline phosphatase) Substrate III kit (Vector).

CAM Binding Assays

To analyze αd/CD18–CAM interactions in solution, 10^6 (per group) αd/CD18 CHO transfectants from suspension cultures were incubated for 20 min at room temperature in 100 μl buffer only (Tris-buffered saline, 0.1% BSA), buffer with 10 μg/ml MAb, or buffer with 2 mM $MgCl_2$. After washing, cells were incubated in buffer only or buffer (± $MgCl_2$) with 10 μg/ml ICAM-3/Ig, ICAM-1/Ig, or VCAM-1/Ig (Sadhu et al., 1994) for 30 min at room temperature. Bound fusion protein was detected with goat anti-human IgG–FITC (Jackson Labs, West Grove, Pennsylvania) diluted 1:100 in buffer, followed by washing and subsequent analysis on a Becton-Dickinson FACscan.

For analysis of integrin binding to immobilized CAM/Ig, microtiter

plates were coated with 10 μg/ml soluble ICAM-3/Ig or ICAM-1/Ig, or 1% BSA and blocked with 1% BSA. CHO transfectants (1×10^5) per group were labeled with 8 μg calcein dye (Molecular Probes, Eugene, Oregon) in phosphate-buffered saline. Calcein-labeled cells were washed and resuspended in buffer with or without 10 μg/ml TS1/18 and incubated at 37°C for 25 min in triplicate wells/group on microtiter plates. After washing by immersion in phosphate-buffered saline with 0.1% BSA for 20 min, remaining fluorescence was determined with an automatic fluorescence reader (Cyto Fluor 2300–2350, Millipore).

Acknowledgments

We thank C. Sadhu, B. Sandmaier, R. Jasman, and P. Hoffman for their advice and support and A. Dersham for manuscript preparation. We greatly appreciate the technical assistance of L. Watson, K. Hensley, and J. Taylor.

Received September 3, 1994; revised October 23, 1995.

References

Anderson, D.C., and Springer, T.A. (1987). Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. *38*, 175–194.

Cole, S.R., Ashman, L.K., and Ey, P.L. (1987). Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol. Immunol. *24*, 699–705.

Collins, S.J. (1987). The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood *70*, 1233–1244.

Corbi, A.L., Miller, L.J., O'Connor, K., Larson, R.S., and Springer, T.A. (1987). cDNA cloning and complete primary structure of the alpha subunit of a leukocyte adhesion glycoprotein, p150,95. EMBO *J. 6*, 4023–4028.

Corbi, A.L., Kishimoto, T.K., Miller, L.J., and Springer, T.A. (1988). The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit: cloning, primary structure, and relation to the integrins, von Willebrand factor and factor B. J. Biol. Chem. *263*, 12403–12411.

Danilenko, D.M., Moore, P.F., and Rossitto, P.V. (1992). Canine leukocyte cell adhesion molecules (LeuCAMs): characterization of the CD11/CD18 family. Tissue Antigens *40*, 13–21.

Danilenko, D.M., Rossitto, P.V., Van der Vleren, M., Trong, H.L., and Moore, P.F. (1995). A novel canine leukointegrin, αdβ2, is expressed by specific macrophage subpopulations in tissues and a minor $CD8^+$ lymphocyte subpopulation in peripheral blood. J. Immunol. *155*, 35–44.

Diamond, M.S., Garcia-Aguilar, J., Bickford, J.K., Corbi, A.L., and Springer, T.A. (1993). The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. Cell Biol. *120*, 1031–1043.

Dustin, M.L., and Springer, T.A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619–624.

Edwards, C.P., Champe, M., Gonzalez, T., Wessinger, M.E., Spencer, S.A., Presta, L.G., Berman, P.W., and Bodary, S.C. (1995). Identification of amino acids in the CD11a I-domain important for binding of the leukocyte function-associated antigen-1 (LFA-1) to intercellular adhesion molecule-1 (ICAM-1). J. Biol. Chem. *270*, 12635–12640.

Ferrante, A., and Thong, Y.H. (1982). Separation of mononuclear and polymorphonuclear leucocytes from human blood by the one-step Hypaque–Ficoll method is dependent on blood column height. J. Immunol. Meth. *48*, 81–85.

Ginsberg, M.H., Du, X., and Plow, E.F. (1992). Inside-out integrin signalling. Curr. Opin. Cell Biol. *4*, 766–771.

Gubler, U., and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene *25*, 263–269.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549–554.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Kishimoto, T.K., O'Connor, K., Lee, A., Roberts, T.M., and Springer, T.A. (1987). Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell *48*, 681–690.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucl. Acids Res. *15*, 8125–8148.

Landis, R.C., Bennett, R.I., and Hogg, N. (1993). A novel LFA-1 activation epitope maps to the I domain. J. Cell Biol. *120*, 1519–1527.

Larson, R.S., and Springer, T.A. (1990). Structure and function of leukocyte integrins. Immunol. Rev. *114*, 181–217.

Larson, R.S., Corbi, A.L., Berman, L., and Springer, T. (1989). Primary structure of the leukocyte function-associated molecule-1 alpha subunit: an integrin with an embedded domain defining a protein superfamily. J. Cell Biol. *108*, 703–712.

Lee, J.O., Rieu, P., Arnaout, M.A, and Liddington, R. (1995). Crystal structure of the A domain from the α subunit of integrin CR3 (CD11b/CD18). Cell *80*, 631–638.

Martin, S., Rieckmann, P., Melchers, I., Wagner, R., Bertrams, J., Voskuyl, A.E., Roep, B.O., Zielasek, J., Heidenthal, E., Weichselbraun, I., Gallatin, W.M. and Kolb, H. (1995). Circulating forms of ICAM-3 (cICAM-3): elevated levels in autoimmune diseases and lack of association with cICAM-1. J. Immunol. *154*, 1951–1955.

Michishita, M., Videm, V., and Arnaout, M.A. (1993). A novel divalent cation-binding site in the A domain of the β2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell *72*, 857–867.

Moore, P.F., Rossitto, P.V., Danilenko, D.M., Wielanga, J.J., Raff, R.F., and Severns, E. (1992). Monoclonal antibodies specific for canine CD4 and CD8 define functional T-lymphocyte subsets and high-density expression of CD4 by canine neutrophils. Tissue Antigens *40*, 75–85.

Randi, A.M., and Hogg, N. (1994). I domain of β2 integrin lymphocyte function-associated antigen-1 contains a binding site for ligand intercellular adhesion molecule-1. J. Biol. Chem. *269*, 12395–12398.

Rojiani, M.V., Finlay, B.B., Gray, V., and Dedhar, S. (1991). In vitro interaction of a polypeptide homologous to human Ro/SS-A antigen (calreticulin) with a highly conserved amino acid sequence in the cytoplasmic domain of integrin alpha subunits. Biochemistry *30*, 9859–9866.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature *362*, 801–809.

Sadhu, C., Lipsky, B., Erickson, H.P., Hayflick, J., Dick, K.O., Gallatin, W.M., and Staunton, D.E. (1994). LFA-1 binding site in ICAM-3 contains a conserved motif and non-contiguous amino acids. Cell Adhes. Comm. *2*, 429–440.

Springer, T.A., Thompson, W.S., Miller, L.J., Schmalstieg, F.C., and Anderson, D.C. (1984). Inherited deficiency of the Mac-1, LFA-1, p150,95 glycoprotein family and its molecular basis. J. Exp. Med. *160*, 1901–1918.

Stone, K.L., McNulty, D.E., LoPresti, M.L., Crawford, J.M., DeAngelis, R., and Williams, K.R. (1992). Elution and internal amino acid sequencing of PVDF-blotted proteins. In Techniques in Protein Chemistry III, R.H. Angeletti, ed. (Bronx, New York: Academic Press, Incorporated), pp. 23–34.

Ueda, T., Rieu, P., Brayer, J., and Arnaout, M.A. (1994). Identification of the complement iC3b binding site in the β2 integrin CR3 (CD11b/CD18). Proc. Natl. Acad. Sci. USA *91*, 10680–10684.

van Kooyk, Y., van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T.W., and Figdor, C.G. (1989). Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature *342*, 811–813.

Vives, J. (1994). CD50 is the "signal transducer" ICAM. The Immunologist *2*, 93–96.

Zhou, L., Lee, D.H., Plescia, J., Lau, C.Y., and Altieri, D.C. (1994). Differential ligand binding specificities of recombinant CD11b/CD18 integrin I-domain. J. Biol. Chem. *269*, 17075–17079.

Gene, 171 (1996) 291–294

GENE 09571

Cloning and chromosomal localization of a novel gene-encoding a human β_2-integrin α subunit

(Recombinant DNA; polymerase chain reaction; *ITGAD*; sequence homology)

Dennis A. Wong[a,1], Elizabeth M. Davis[b], Michelle LeBeau[b] and Timothy A. Springer[a,*]

[a]*The Center For Blood Research, Harvard Medical School, Boston, MA 02115, USA;* [b]*Department of Medicine, University of Chicago, 5841 South Maryland Avenue, MC2115, Chicago, IL 60637-1990, USA. Tel. (1-312) 7029118*

Received by J.K.C. Knowles: 13 March 1995; Revised/Accepted: 16 August/22 August 1995; Received at publishers: 11 December 1995

SUMMARY

We isolated a partial genomic clone encoding ITGAD, a novel β_2-integrin α subunit. The *ITGAD* gene is highly homologous to the three previously known α subunit-encoding genes, that compose the β_2 integrin family, in deduced amino acid sequence, intron/exon structure and mapping location (chromosome 16p11).

INTRODUCTION

Integrins (Itg) are a large family of cell surface αβ heterodimers involved in cell-cell and cell-extracellular matrix interactions (Hynes, 1992). Three Itg that share the β_2 (CD18) subunit are restricted in expression to leukocytes and have homologous α subunits: α^L (LFA-1, CD11a), α^M (Mac-1, CD11b), and α^X (p150/95, CD11c) (Larson and Springer, 1990). These Itg are important in the emigration of leukocytes from the vasculature, interaction with target cells and antigen-presenting cells, and binding to iC3b and fibrinogen (Springer, 1995). Inherited defects in the β_2 subunit, which lead to the absence of these three heterodimers in leukocyte adhesion deficiency, result in life-threatening bacterial infections (Anderson and Springer, 1987).

To study the possibility of an alternative Itg α subunit which could associate with β_2, a genomic molecular cloning approach was used and a novel gene was found.

EXPERIMENTAL AND DISCUSSION

(a) The cloning of a novel *ITG* gene

A genomic approach was used, because the genome would contain all α subunit-encoding genes, regardless of cellular distribution, in equal numbers. The most conserved region of α^L, α^M and α^X is in the EF hand-like putative divalent cation binding repeats. To identify novel leukocyte integrin α subunits, a number of degenerate PCR primers complementary to this region were designed based on the known intron/exon structure of the human α^X-encoding gene, *ITGAX* (Corbi et al., 1990). primers A, 5′-GGGRGCMCCYCGMTAYCAGCACA; B, 5′-ATYGGCKCYTAYTTCGGKG; W, 5′-ASAYRRACACCYGGCCYCCTC; and X, 5′-CTCCTCYMSWGGGGCCCCIAYRRCCACGTC (where K = G or T; M = A or C; R = A or G; S = C or G; Y = C or T) were able to generate PCR products of the appropriate length. Using the exon numbering system for *ITGAX*, primers

* *Correspondence to:* Dr. T.A. Springer, The Center for Blood Research, Room 251, 200 Longwood Avenue, Boston, MA 02115, USA. Tel. (1-617) 278-3200; Fax (1-617) 278-3232; e-mail: springer@cbrv1.med.harvard.edu

[1] Current address: Division of Rheumatology, Allergy, and Immunology, McGuire Hall, Room 4-110, 1112 East Clay Street, P.O. Box 980263, Richmond, VA 23298-0263, USA. Tel. (1-804) 828-9685.

Abbreviations: aa, amino acids(s); bp, base pair(s); cDNA, DNA complementary to RNA; iC3b, inactivated third component of complement b fragment; Itg, integrin(s); *ITG*, gene encoding Itg; *ITGAD*, gene encoding a novel Itg α^D subunit; *ITGAX*, gene encoding the Itg α^X subunit; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; RT, reverse transcription(tase).

SSDI 0378-1119(95)00869-1

B and W were located within exon 14. PCR products from genomic DNA using primers B and W were cloned and sequenced and included known leukocyte integrin α subunit gene sequences as well as products that were most similar to *ITGAX*, but differed by 5 nt. The products of primers A (from exon 13) and X (from exon 15) included *ITGAX* and a product that corresponded to the novel BW product in exon 14 and was homologous to known α subunit genes in the exons, but differed substantially in the two introns.

The intron between exons 14 and 15 of this novel product was used to probe 5×10^5 clones of a human pWE15 genomic cosmid library (kindly provided by Glen Evans, Salk Institute). A clone with a 37-kb insert designated pWE15αW.1 was isolated.

(b) Sequencing a novel *ITG* gene and aa comparison

Sequences that hybridized with the full-length α^X and α^M cDNAs were localized to a 21-kb *Not*I-*Eco*RI fragment on the 5′ end of the cosmid insert. *Sau*3A, *Taq*I, *Hae*I and *Bgl*II fragments that hybridized with the α^X cDNA were selected from this region for sequencing. Over 5000 nt were sequenced, including a 4153-nt fragment containing exons 25–30. Eleven putative exons were identified by sequence homology (Fig. 1A). A twelfth exon, exon 14, was sequenced in the PCR fragment, but did not appear in the cosmid clone, in which the intron between exons 14 and 15 is at the 5′ end. Seven exons were completely sequenced (21 and 25–30) and five were sequenced through one intron boundary. Exons 18–20, 22, and 23 have not been localized and are predicted to be present in regions that remain to be sequenced. All exons thus far identified contained ORFs, consensus splice sites, and the same intron phase as in the α^X (Corbi et al., 1990) and α^M (Fleming et al., 1993) genes.

The exons correspond to the C-terminal two-thirds of the putative a subunit, from the three EF hand-like putative divalent cation binding repeats to the transmembrane domain. The translated aa sequence is most homologous to the α^X subunit (70% identity) compared with α^M (59%) and α^L (32%) (Fig. 1B). We designate this novel gene *ITGAD* and predict it is expressed as a novel β_2 integrin α^D subunit. Nested RT-PCR on tonsillar RNA using primer BW, 5′-GACAGCGATGGCAGCACCGACCTGA, derived from the *ITGAD* exon 14 sequence and primer X after an initial round of PCR with primers A and X, showed the novel gene to be processed at least to the point of having the intron between exons 14 and 15 spliced out. This 220-nt product when subcloned and sequenced was 86% identical to the α^X sequence, as compared to the 92% identity of α^X and α^M over the same region. Using this product as a probe and by combining RT-PCR and enrichment using specific restriction enzymes, the predicted transcription product of this gene could be shown to be present in tonsilar mRNA (Fig. 2). Furthermore another group of investigators have independently, using an alternative strategy, cloned the cDNA of an α subunit with essentially identical sequence to the predicted sequence from our gene (W.M. Gallatin, unpublished personal communication). Our mutual use of the letter D for this integrin is based on the likelihood that the protein will be designated CD11d in the future.

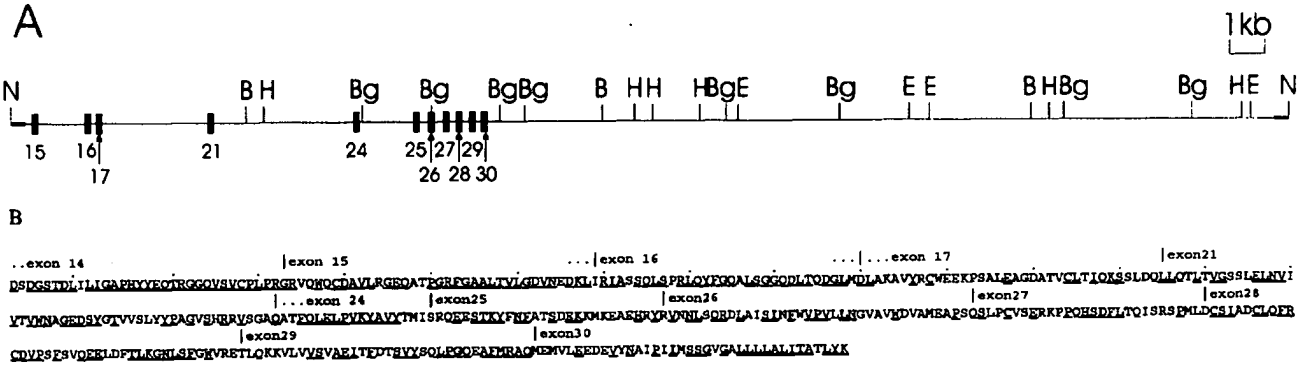


Fig. 1. Organization and partial sequence of *ITGAD*. A: Genomic organization. Restriction map of the human genomic 37-kb pWE15αW.1 cosmid insert to an accuracy of 250 bp. *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), and *Not*I (N). Filled boxes represent possible exons that are numbered based on homology to the exons of *ITGAX* (Corbi et al., 1990). Thicker lines indicate flanking vector sequence containing *Not*I sites. Mapping was done by Southern hybridization of digested fragments with ^{35}S-labeled exons, as well as PCR between exons and between the T3 promoter site (at the left or 5′ end) and certain exons. B: Homology of the translated putative exons of *ITGAD* with the α^L (CD11a), α^M (CD11b), and α^X (CD11c) subunits are underlined when shared by two of the other subunits (GenBank accession Nos.: U40274, U40275, U40276, U40277, U40278, U40279). Translated exons from α^D were combined for alignment with the corresponding segments from other leukocyte integrin subunits. Exon boundaries are indicated by vertical lines and three periods indicate segments that do not extend fully to the predicted boundary. Segments used for alignment and Swiss-prot database accession Nos. were: α^L, aa 470–537, 567–600, 631–662, 789–836, and 910–1113 (P20701); α^M, aa 467–537, 571–602, 633–666, 790–836, and 914–1130 (P11215); α^X, aa 468–537, 571–602, 633–666, 790–836, and 912–1129 (P20702).

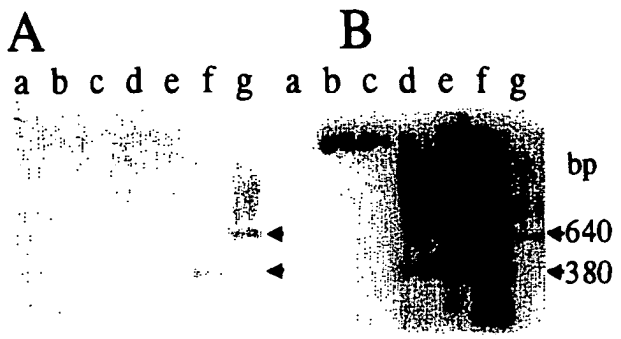


Fig. 2. *ITGAD* transcription product present in tonsilar RNA after enrichment. **A**: To detect transcription of the novel gene, tonsilar mRNA was submitted to RT-PCR with degenerate primers A and X. The RNA was then enriched for the novel sequence by digesting with three specific restriction enzymes *FokI*, *SalI*, and *NarI* which cut the known α subunit cDNAs in this region and then resubmitted for PCR with A and X. Only after two rounds of this type of enrichment could the appropriate 380-bp product be detected with the ^{32}P-labeled 220-bp probe from the new sequence. In genomic DNA where the novel gene sequence would be expected to be expressed in equal amounts to the other α subunits the appropriate 640-bp product with the two introns still present is readily detected. **B**: In contrast, α ^{32}P-labeled probe from the same region of α^M is able to detect α^M sequence without enrichment on the same blot. The 92% homology between α^M and α^X over this region leads to some cross-reactivity between the α^M probe and α^X. An equal number of copies of sequence was loaded on a 1.2% agarose gel in each lane by weighting for the expected length of an individual copy. Lanes: a, cDNA α^L; b, cDNA α^M; c, cDNA α^X; d, RT-PCR product of degenerate primers A and X; e, PCR product of primer A and X after triple digestion; f, PCR product of primer A and X after a second round of triple digestion; g, PCR product of primers A and X using genomic DNA as a template.

(c) The chromosomal localization of *ITGAD*

To localize the *ITGAD* gene, we performed fluorescence in situ hybridization with a biotin-labeled probe of the cosmid insert on normal human metaphase chromosomes (Rowley et al., 1990). Specific labeling of chromosome 16, band p11 (Fig. 3) was observed on four chromatids (16 cells), three chromatids (7 cells) or two chromatids (2 cells) in 25 cells examined. A single background signal was observed on 3p12. Localization to 16p11 was confirmed in a second hybridization experiment. *ITGAD* thus clusters with the α^L, α^M, and α^X genes, which also localize to chromosome 16, band p11 (Corbi et al., 1988).

(d) Conclusions

The similarity in gene organization, sequence, and chromosomal location suggest that *ITGAD* encodes a fourth leukocyte integrin α subunit. This subunit is much more homologous to the three β_2 integrin α subunits than to other integrin α subunits, and may associate with the β_2 subunit and be expressed on leukocytes. Previous studies have shown a subunit lower in M_r than α^L, α^M, or α^X that associates with β_2 on human monocytes (Sanchez-Madrid et al., 1983), and an α subunit that associates with β_2 on canine macrophages and lymphocytes that appears distinct from α^L, α^M, and α^X (Danilenko et al., 1990). The relationship of these subunits to α^D remains to be characterized.

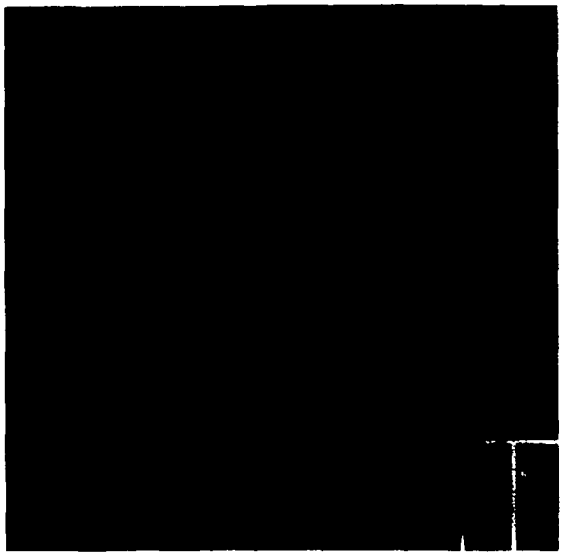


Fig. 3. Chromosome localization. The chromosome 16 homologues are identified with arrowheads. The inset shows partial karyotypes of two chromosome 16 homologues illustrating specific labeling at 16p11 (arrow). A biotin-labeled human *ITGAD* cosmid probe was prepared by nick translation using Bio-11-dUTP (Enzo Diagnostics) and used in fluorescence in situ chromosomal hybridization with metaphase phytohemogglutin-stimulated peripheral blood lymphocytes as described (Rowley et al., 1990). Hybridization was detected with flouresceinconjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride.

ACKNOWLEDGMENTS

Supported by NIH grants CA31799 and CA40046. D.A.W. was supported by an MRC of Canada Clinician Scientist Award. We thank Lloyd Klickstein for advice and Steven Minaglia for assistance with chromosomal localization.

REFERENCES

Anderson, D.C. and Springer, T.A.: Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. Annu. Rev. Med. 38 (1987) 175–194.

Corbi, A.L., Larson, R.S., Kishimoto, T.K., Springer, T.A. and Morton, C.C.: Chromosomal location of the genes encoding the leukocyte adhesion receptors LFA-1, Mac-1 and p150,95. Identification of a gene cluster involved in cell adhesion. J. Exp. Med. 167 (1988) 1597–1607.

Corbi, A.L., Garcia-Aguilar, J. and Springer, T.A.: Genomic structure of an integrin alpha aubunit, the leukocyte p150,95 molecule. J. Biol. Chem. 265 (1990) 2782–2788.

Danilenko, D.M., Moore, P.F. and Rossitto, P.V.: Canine leukocyte

cell adhesion molecules (LeuCAMs): characterization of the CD11/CD18 family. Tissue Antigens 40 (1992) 13–21.

Fleming. J.C., Pahl, H.L., Gonzealez, D.A., Smith, T.F. and Tenen, D.G.: Structural analysis of the CD11b gene and phylogenetic analysis of the α-integrin gene family demonstrate remarkable conservation of genomic organization and suggest early diversification during evolution. J. Immunol. 150 (1993) 480–490.

Hynes, R.O.: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69 (1992) 11–25.

Larson, R.S. and Springer, T.A.: Structure and function of leukocyte integrin. Immunol. Rev. 114 (1990) 181–217.

Rowley, J.D., Diaz, M.O., Espinosa, R., Patel, Y.D., Van Melle, E., Ziemin, S., Taillon-Miller, P., Lichter, P., Evans, G.A., Kersey, J.D., Ward, D.C., Domer, P.H. and LeBeau, M.M.: Mapping chromosome band 11q23 in human acute leukemia with biotinylated probes: identification of 11q2323 translocation breakpoints with a yeast artificial chromosome. Proc. Natl. Acad. Sci. USA 87 (1990) 9358–9362.

Sanchez-Madrid, F., Nagy, J., Robbins, E., Sinon, P. and Springer, T.A.: A human leukocyte differentiation antigen family with distinct alpha subunits and a common beta subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. J. Exp. Med. 158 (1983) 1785–1803.

Springer, T.A.: Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu. Rev. Physiol. 57 (1995) 827–872.

APPENDIX 5

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Klein et al., J Cell Biol. 1991 Dec;115(5):1427-36. (10 pages)

Gardner et al., Dev Biol. 1996 May 1;175(2):301-13. (13 pages)

Integrin $\alpha_2\beta_1$ Is Upregulated in Fibroblasts and Highly Aggressive Melanoma Cells in Three-Dimensional Collagen Lattices and Mediates the Reorganization of Collagen I Fibrils

C. Eberhard Klein,* Daniela Dressel,* Thomas Steinmayer,* Cornelia Mauch,§ Beate Eckes,§ Thomas Krieg,§ Richard B. Bankert,‡ and Lutz Weber

*Department of Dermatology, University of Ulm, Ulm, Germany; ‡Department of Molecular Immunology, Roswell Park Memorial Institute, Buffalo, New York 14263; and §Department of Dermatology, Ludwig-Maximilians-University Munich, Munich, Germany

Abstract. The ability of cultured human fibroblasts to reorganize and contract three dimensional collagen I gels is regarded as an in vitro model for the reorganization of connective tissue during wound healing. We investigated whether adhesion receptors of the integrin family are involved. It was found that synthesis and transcription of the $\alpha_2\beta_1$ integrin (but not of $\alpha_1\beta_1$ or $\alpha_3\beta_1$) is selectively upregulated when fibroblasts are seeded into type I collagen gels. Time course experiments revealed that high synthetic levels of $\alpha_2\beta_1$ parallel the gel contraction process and return to "baseline" levels after the contraction has subsided. Furthermore, function-blocking mAbs directed to the α_2 and β_1 chain of integrins inhibited gel contraction.

Remodelling of connective tissue can be important for tumor cells during invasion and formation of metastases. Therefore, we tested human melanoma cell lines for this function. Five out of nine melanoma lines contracted collagen gels in vitro. Among these, two highly aggressive melanoma cell lines (MV3 and BLM) most efficiently contracted gels almost reaching the rate of normal adult fibroblasts. In these cells, synthesis of $\alpha_2\beta_1$ was also significantly upregulated when seeded into collagen I gels. Moreover, function blocking anti-α_2 in conjunction with anti-β_1 chain mAbs completely inhibited gel contraction for several days. Other melanoma cells (530) with lower metastatic potential which were not able to contract gels, showed no induction of $\alpha_2\beta_1$ synthesis in gel culture. Our results suggest an important role of integrin $\alpha_2\beta_1$ in the contraction of collagen I by normal diploid fibroblasts during wound healing and in the reorganization of collagen matrices by highly aggressive human melanoma cells.

THE reorganization of collagen by fibroblasts is an important function in wound healing which leads to wound contraction and finally helps to reestablish organ integrity. The ability of cultured fibroblasts to reorganize and contract three-dimensional collagen I gels (Bell et al., 1979) is considered as an in vitro model for wound contraction. Previous studies have described in detail the influence of cytokines (Gullberg et al., 1990), the requirement of protein synthesis and of an intact cytoskeleton for this process (Mauch, 1986; Guidry and Grinnell, 1985). Seeding of fibroblasts into a three-dimensional collagen lattice results in major changes of their morphology (Tomasek et al., 1982), their protein and collagen metabolism (Mauch et al., 1988) as well as in their response to cytokines (Nagakawa et al., 1989). However, little is known, so far, about the role of extracellular matrix (ECM)[1] receptors on the fibroblast surface for this function. Recently, evidence has been provided that polyclonal antisera directed against the β_1 chain of integrins may interfere with gel contraction (Gullberg et al., 1990). Among the β_1 subgroup of integrins, at least three receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$) are known to interact with collagen (Wayner and Carter, 1987; Belkin et al., 1990; Kirchhofer et al., 1990).

In this study, our first aim was to identify single integrin receptors involved in this process. We show that $\alpha_2\beta_1$ is the only integrin which is strongly upregulated when fibroblasts start contracting collagen I gels. Furthermore, we demonstrate that function blocking anti-α_2- in conjunction with anti-β_1-chain mAbs most efficiently inhibit gel contraction.

The capability to reorganize collagen may also be advantageous for tumor cells during tissue invasion. Recent studies revealed that $\alpha_2\beta_1$ is involved in the migration of tumor cells within collagenous matrices (Yamada et al., 1990) and that it is expressed at increased frequency during tumor progression in human melanoma (Klein et al., 1991). Therefore, we also tested human melanoma cell lines for their ability to reorganize collagen I lattices and investigated the role of $\alpha_2\beta_1$ in this function.

1. *Abbreviations used in this paper*: CFN, cellular fibronectin; ECM, extracellular matrix.

Material and Methods

Cells and Culture Conditions

Cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 1% nonessential aminoacids, 100 U/ml penicillin, and 100 U/ml streptomycin. Cultures of normal fibroblasts and melanocytes were established and maintained as previously described (Klein et al., 1988; Eisinger and Marko, 1982; Halaban et al., 1986). Melanocyte cultures (M.LM, p-5; M.SD, p-12; M.RF, p-6; and M.HF, p-6) established from the foreskin of young children or young adults were kindly provided by Dr. D. Kaufman (Department of Human Genetics, University of Ulm, Ulm, Germany). The fetal fibroblast cell line F 135-60-86-skin was originally obtained from Dr. J. Fogh's cell bank at the Memorial Sloan Kettering Cancer Center, New York. The melanoma cell lines SK-MEL-13, -19, -29, and -113 were gifts from Dr. L. J. Old (Memorial Sloan Kettering Cancer Center, New York). The melanoma cell lines 530 (Versteeg et al., 1988), BLM (van Muijen et al., 1989), and MV3 (van Muijen et al., 1991) were kindly provided by Dr. G. van Muijen (Department of Pathology, Academish Ziekenhuis, University of Nijmwegen). The squamous carcinoma cell lines SCL1 and -2 (Tilgen et al., 1986) were gifts from Dr. N. E. Fusenig (Department of Biochemistry, German Cancer Research Center, Heidelberg, Germany). All cell lines were repeatedly subjected to hybridization tests using ^{3}H-labeled mycoplasma DNA (Mycoplasma TC Gen Probe Inc., San Diego, CA) and were negative.

Monoclonal antibodies

Serum or ascites of hybridoma bearing mice or tissue culture supernatant was the source of mAbs: mAb TS2/7 binds to the α_1-chain of integrin $\alpha_1\beta_1$ (VLA-1) (Hemler et al., 1985), mAbs 10G11 (Hemler et al., 1988), A-1-43 (Klein et al., 1991), Gil4 (Santoso et al., 1989), 5E8 (Zylstra et al., 1986; Takada et al., 1989), and P1E6 (Wayner et al., 1988) detect the α_2 chain of $\alpha_2\beta_1$, mAbs J143 (Kantor et al., 1987), and P1B5 (Wayner and Carter, 1987) define the α_3 chain of $\alpha_3\beta_1$. mAbs B5G10 (Hemler et al., 1987) and P4G9 (Wayner et al., 1989) recognize the α_4 chain of $\alpha_4\beta_1$; mAbs 16 (Akyama et al., 1989) and P1D6 (Wayner et al., 1988) bind to the α_5 chain; mAbs GoH3 (Sonnenberg et al., 1989) and MT78 (Klein et al., 1990) detect the α_6 chain; mAb LM142 (Cheresh and Spiro, 1987) recognizes the α_v chain. mAbs Aj2 (Kantor et al., 1987), 13 (Akyama et al., 1989), and 4B4 (Morimoto et al., 1985; Shimizu et al., 1990) are directed to the β_1 chain. mAbs TS2/7 and B5G10 were kindly provided by M. Hemler (Dana Farber Cancer Center, Boston, MA); the purified mAbs 16 and 13 were gifts from S. Akayama (Howard University Cancer Center, Washington, DC). mAbs J143 and Aj2 were kindly provided by L. J. Old (Memorial Sloan Kettering Cancer Center, New York) mAbs GoH3 and 10G11 were gifts from A. Sonnenberg (Central Blood Bank of the Netherlands, Amsterdam). mAb LM142 was kindly provided by D. Cheresh (Scripps Clinic, La Jolla, CA). mAbs P1E6, P1D6, P4G9, and 4B4 were purchased from Telios Pharmaceuticals Inc. (San Diego, CA) and Coulter Corporation (Hialeah, FL), respectively. The function blocking mAbs used in this study are listed in Table I. For inhibition studies, mAbs were purified by ammonium sulfate precipitation and subsequent affinity chromatography on PA-Sepharose Columns using standard procedures.

Preparation of Collagen Gels (Hydrated Collagen I Lattices)

Collagen I was extracted from rat tail tendons and stored lyophilized as previously described (Mauch et al., 1988). Collagen I (2 mg/ml) was dissolved in 0.1% acetic acid and stored at 4°C as stock solution. For gel preparation, 1.85 ml Mc Coy's medium (1.95-fold concentrated), 0.9 ml FBS, 0.25 ml 0.1 N NaOH was added to 1.5 ml collagen I stock solution in 60-mm bacteriological petri dishes and carefully mixed by circular movements. Then, 1×10^6 cells suspended in 0.5 ml Mc Coy's medium containing 20% FBS were added and the solution (5 ml) was mixed again. Gel formation occurred within the first two hours of culture at 37°C and 5% CO_2. For experiments in 35-mm bacteriological petri dishes, gels of 2-ml vol containing 4×10^5 cells were prepared. For inhibition studies, purified mAbs were added during gel preparation. The mAb concentration in the stock solutions added ranged from 0.6 to 1.6 mg/ml (0.1 M Tris, pH 8).

Radioimmunoprecipitation

Cells in monolayer or gel culture were metabolically labeled with [^{35}S]methionine (60–200 μCi/ml; New England Nuclear, Boston, MA) for 6 or 16 h in methionine-free medium containing 10% dialyzed FBS. After metabolic labeling, the collagen gels were immersed into NP40-lysis buffer (0.5% NP-40, 0.015 M NaCl, 0.01 M Tris pH 7.5, 0.002 M PMSF, and Aprotinin), minced into fine pieces, repeatedly aspirated into syringes, and forced through needles with decreasing diameter. Monolayer cultures were incubated with NP-40 lysis buffer, scraped off the tissue culture plastic ware and then treated equally as the gel culture cell lysate. Glycoproteins were isolated from NP-40 solubilized cell extracts by adsorption to Con A Sepharose (Pharmacia Inc., Uppsala, Sweden) (Lloyd et al., 1981). Immunoprecipitations were carried out as previously described (Klein et al., 1988). To compare glycoprotein synthesis under different culture conditions, equal numbers of counts of the Con A–bound fractions were immunoprecipitated. The amounts of precipitated glycoproteins were determined after SDS-PAGE by quantitative density scanning of the fluorographs.

Table I. Function Blocking mAbs Directed to Integrins Used in This Study

Polypeptide	mAb	Reference
α_2 chain	5E8	Zylstra et al., 1986
		Takada et al., 1989
	P1E6	Wayner et al., 1988
α_3 chain	P1B5	Wayner et al., 1987
α_5 chain	16	Akyama et al., 1989
β_1 chain	13	Akyama et al., 1989
	4B4	Shimizu et al., 1990
		Morimoto et al., 1985

RNA Isolation and Northern Blot Hybridization

Total RNA was isolated from fibroblasts as described previously (Mauch et al., 1988). Briefly, cells were homogenized in 4 M guanidinium isothiocyanate using a Potter-Elvehjem and extracted with phenol/chloroform to remove collagenous debris. The supernatants were then centrifuged through a 5.7 M CsCl cushion. The RNA pellet was dissolved in water, treated with phenol/chloroform, precipitated with 0.3 M sodium acetate, and 2.5 vol of ethanol. For Northern blot hybridization, 5 μg of total RNA was separated by electrophoresis on a 1% formaldehyde agarose gel and transferred to Gene Screen hybridization transfer membranes (New England Nuclear Research Products, Boston, MA). The filters were cross-linked by UV (Stratagene) and hybridized with prime-labeled radioactive cDNA probes specific for the VLA α_2 and β_1 chain as well as for β tubulin. Densitometric scans were performed from autoradiographs to quantify the intensity of hybridization (Hirschmann Elscript 400). cDNA's for VLA α_2 (clone 2.72L) (Takada and Hemler, 1989) were obtained from Dr. M. E. Hemler (Dana Farber Cancer Center, Boston, MA) for VLA β_1 (clone p GEM1-P32) (Argraves et al., 1987) from Dr. E. Ruoslahti (La Jolla Cancer Research Institute, La Jolla, CA) and for β-tubulin (clone Dβ_1) (Hall et al., 1983) from Dr. D. W. Hall (Dept. of Biochemistry, New York University, New York).

Results

When seeded into collagen I gels, fibroblasts contract and reorganize the collagen leading to the formation of a dense "interstitial connective tissue." For our studies, adult human dermal fibroblasts were seeded at a density of 2×10^5 cells/ml into gels containing 0.6 mg/ml collagen I which had been purified from rat tail tendons. Time course studies showed that under this condition contraction of the gels was first noticeable after 6–7 h. Most of the contraction, however, occurred between 12 and 48 h and after 72–80 h the process was completed. A representative experiment is shown in Fig. 1.

First, we compared the synthesis of integrins in fibroblast monolayer cultures with that of split cultures which had been seeded into the gels. Cells were metabolically labeled with

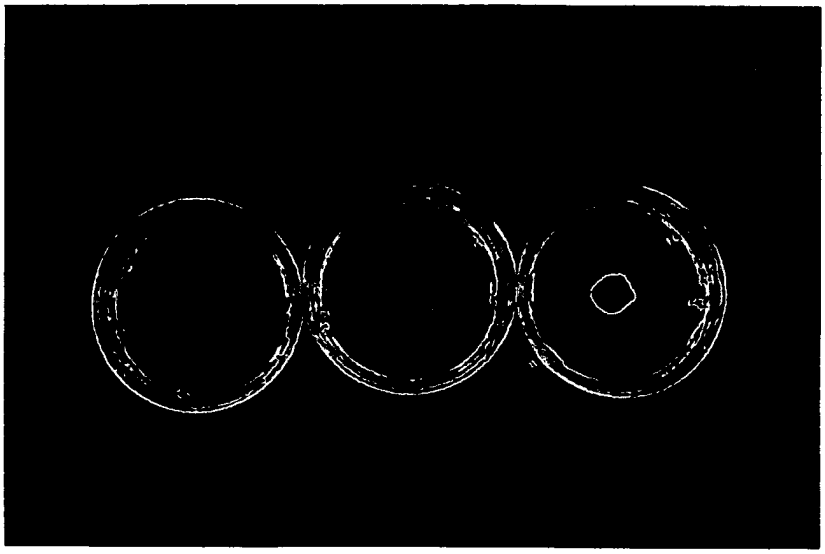


Figure 1. Contraction of collagen I gels by human adult fibroblasts (H-45). Three collagen I gel cultures (2 ml) of 2 × 10^5 human adult fibroblasts (H-45) were prepared in 35-mm bacteriological petri dishes. Cultures were photographed at different times after culture initiation. From left to right: 6, 18, 48 h.

[^{35}S]methionine for 16 h, starting 24 h after culture initiation. Synthesis of integrin receptors was assessed by immunoprecipitation from the Con A–bound fractions of NP-40 cell lysates. mAbs directed to the α_1-, α_2-, α_3-, α_4-, α_5-, α_6-, α_v, and β_1 chain of integrins were used.

Fibroblasts contracting collagen gels showed 10- to 14-fold higher synthetic levels of $\alpha_2\beta_1$ than monolayer cultures, whereas all other integrins studied were unchanged ($\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_x$) or reduced ($\alpha_4\beta_1$) (Figs. 2 and 3). It was remarkable that the two other β_1 integrins which are known to also bind collagen were unchanged. To exclude the possibility that changes in the glycosylation of integrins led to their differential binding to Con A we also immunoprecipitated the seven receptors from whole cell lysates of the two culture conditions without adsorption to Con A and found the same relationship as between the immunoprecipitates from the Con A–bound glycoproteins (not shown). Particularly, the same 10- to 14-fold increase of the $\alpha_2\beta_1$ immunoprecipitate in fibroblasts contracting collagen gels was observed. Furthermore, the comparison of the immunoprecipitates from whole cell lysates and from the Con A–bound fractions by SDS-PAGE revealed the same electrophoretic mobility suggesting that there are no major differences in glycosylation. In respect to $\alpha_2\beta_1$, the results were further confirmed by comparative immunoprecipitations from monolayer and gel cultures using three different mAbs (10G11,

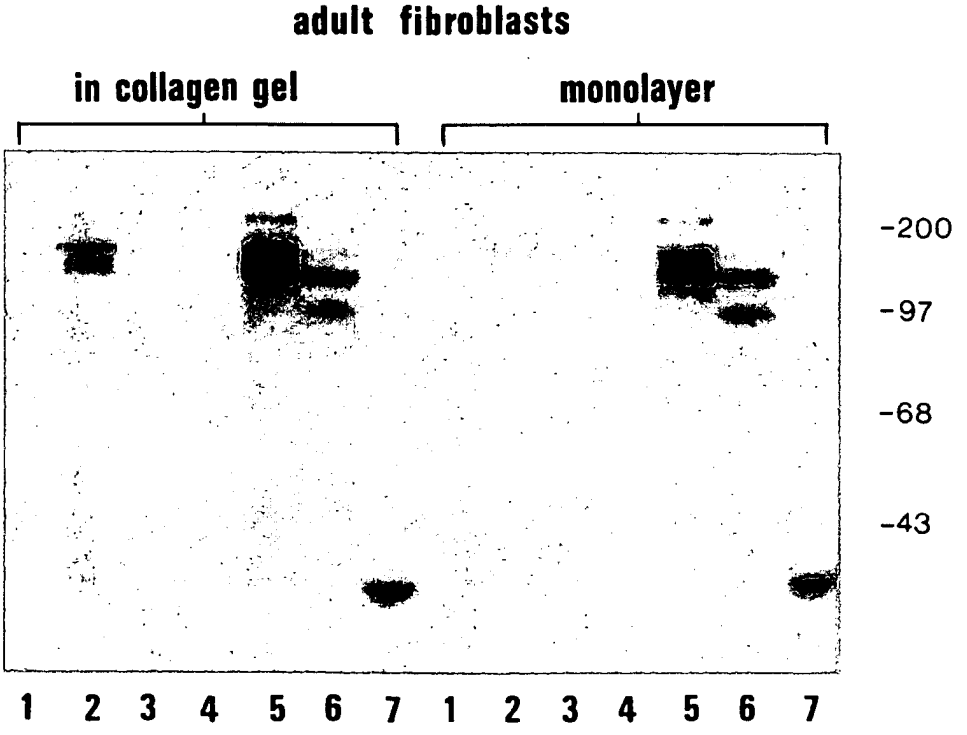


Figure 2. Synthesis of integrins in collagen I gel and monolayer cultures of normal human fibroblasts. Con A-bound fractions of NP-40 cell lysates from [^{35}S]methionine-labeled parallel cultures (H-45) were analyzed by immunoprecipitation and SDS-PAGE. The fluorograph shows immunoprecipitates obtained with different mAbs: (lane *1*) control, normal mouse serum; (lane *2*) 10G11, anti-α_2; (lane *3*) J143, anti-α_3; (lane *4*) GoH3, anti-α_6; (lane *5*) Aj2, anti-β_1; (lane *6*) LM 142, anti-α_v; (lane *7*) W6/32, anti-HLA class I.

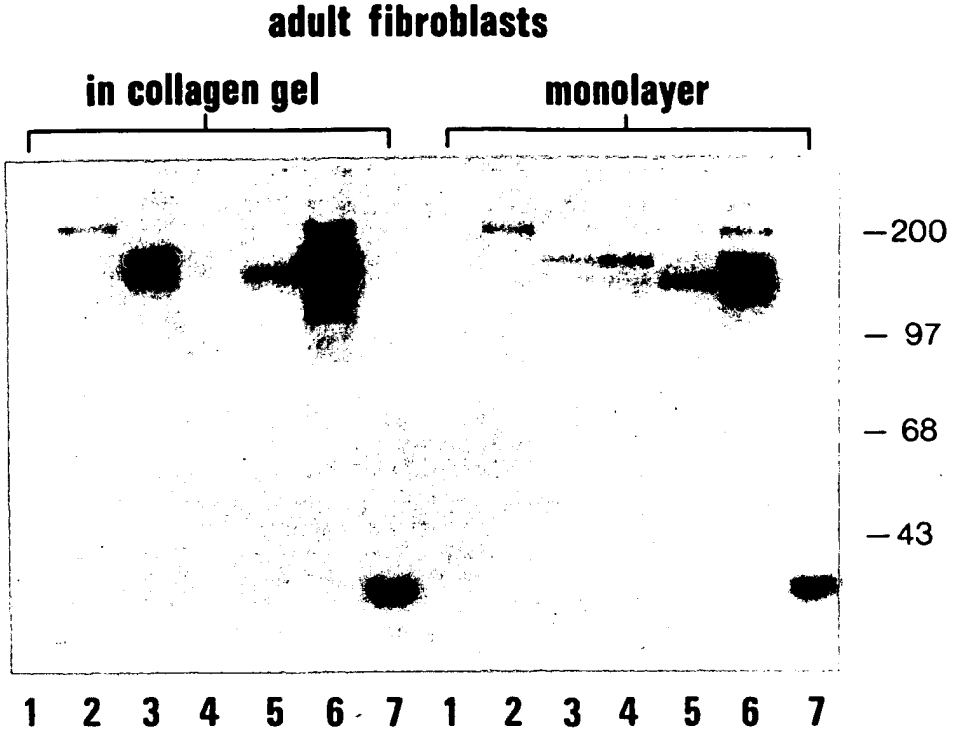


Figure 3. Synthesis of integrins in collagen I gel and monolayer cultures of normal human fibroblasts. Con A-bound fractions of NP-40 cell lysates from [^{35}S]methionine-labeled parallel cultures (H-45) were analyzed by immunoprecipitation and SDS-PAGE. The fluorograph shows immunoprecipitates with different mAbs: normal mouse serum (lane *1*); Ts2/7, anti-α_1 (lane *2*); 10G11, anti-α_2 (lane *3*); B5G10, anti-α_4 (lane *4*); P1D6, anti-α_5 (lane *5*); Aj2, anti-β_1 (lane *6*); and W6/32, anti-HLA class I (lane *7*).

Gi14, and 5E8). These mAbs define at least two nonoverlapping epitopes on the α_2 subunit (unpublished data). To insure that the differences of integrin synthesis were not caused by other experimental artifacts, we also compared the synthesis of HLA class I proteins using mAb W6/32 which defines a monomorphic determinant of the molecules. No change of the synthetic levels of this glycoprotein was noticeable under both culture conditions (Figs. 2 and 3). The selective induction of $\alpha_2\beta_1$ synthesis was also seen when fetal skin fibroblasts were seeded into collagen lattices. Here, the synthetic levels of $\alpha_2\beta_1$ differed in monolayer and gel culture by the same ratio (1:10–14) as found for adult fibroblasts (not shown).

Time course studies were performed to further analyze the upregulation of $\alpha_2\beta_1$. Adult fibroblasts in "gel culture" were metabolically labeled for 6 h at different times after culture initiation: it was found that $\alpha_2\beta_1$ synthesis was already increased when cells were labeled 6 h after initiation of the cultures. High levels of $\alpha_2\beta_1$ synthesis were seen at 6–12, 24–30, and 48–54 h with the peak level between 24–30 or 48–54 h. Interestingly, at day 5 when gel contraction had subsided, the synthetic activity of $\alpha_2\beta_1$ was decreased to the "baseline" level of monolayer fibroblasts (Fig. 4). HLA class I antigens (mAb W6/32) were analyzed in parallel for each condition and no change of their synthetic levels was noted (not shown). In some fibroblast cell lines, which showed a particular high contraction rate, the level of $\alpha_2\beta_1$ synthesis already declined 24 h after culture initiation. From these ex-

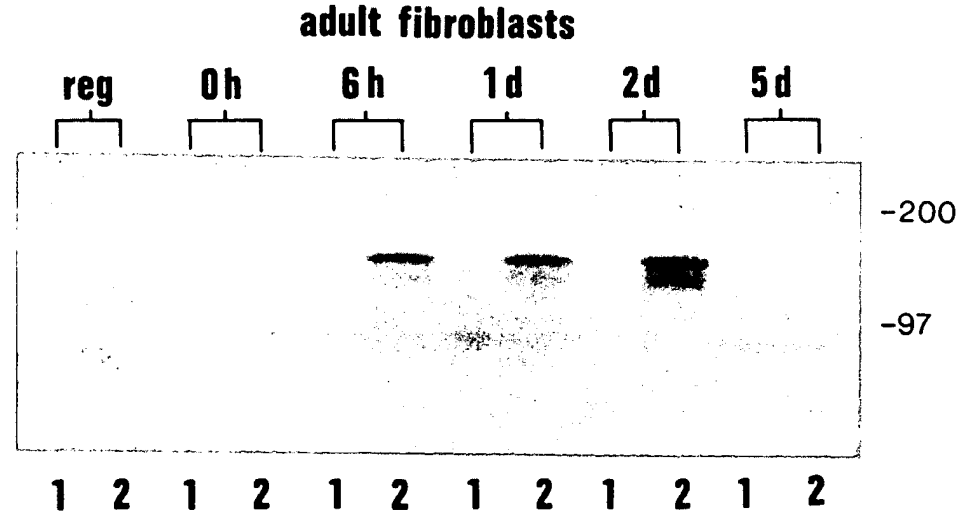


Figure 4. Synthesis of integrin $\alpha_2\beta_1$ by fibroblasts in gel cultures. Adult fibroblasts (H-45) in collagen I gel culture were metabolically labeled for 6 h at different times after culture initiation (0, 6, 24, 48 h, and 5 d). The synthetic levels of $\alpha_2\beta_1$ were then compared with that of fibroblast monolayer cultures (*reg*). Cell lysates were processed for immunoprecipitation as described in Materials and Methods. (lane *1*) normal mouse serum, control; (lane *2*) mAb 10G11, anti-α_2 chain.

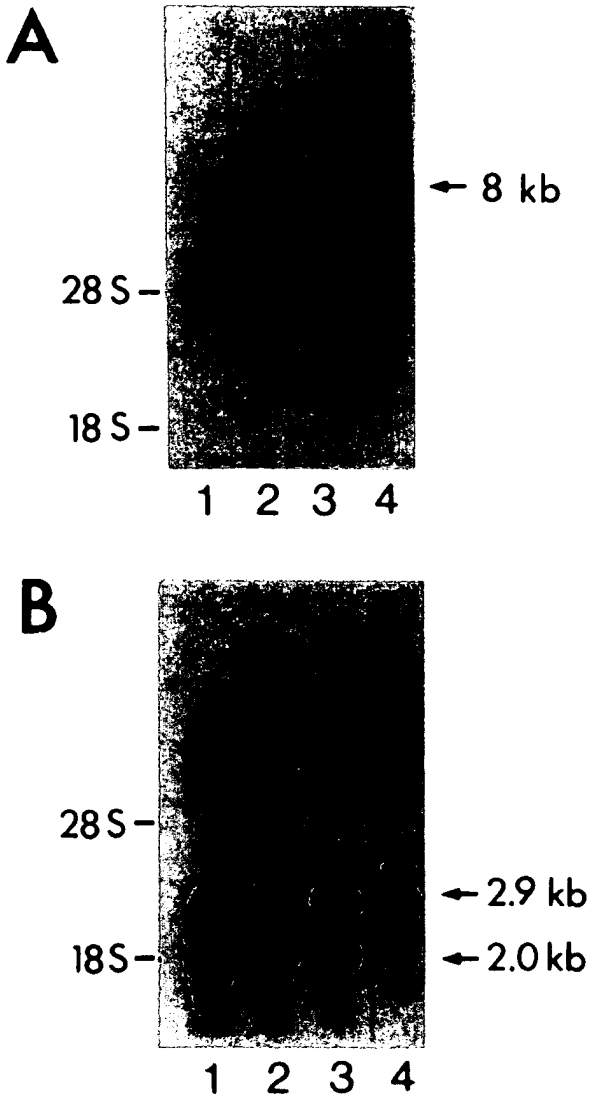


Figure 5. Expression of VLA-α_2 by cells in collagen gels and monolayers. Total RNA was isolated from fibroblasts (NH-1) grown for different times in collagen gels (*2–4*) and as monolayers (*1*). Total RNA was separated in a denaturing agarose gel containing 1% formaldehyde, blotted and hybridized with the radioactively labeled cDNA probe specific for VLA-α_2 (*A*) and β-tubulin (*B*). Positions of 18S and 28S RNA markers are indicated. Lane *1* represents mRNA from fibroblasts grown for 24 h as monolayers, lanes *2–4* mRNA from fibroblasts grown for different times in collagen gels (6, 12, and 18 h).

periments it was concluded that high levels of $\alpha_2\beta_1$ synthesis are coincidentally associated with the process of gel contraction. We then analyzed the transcriptional regulation of α_2 and β_1 chain expression. mRNA was extracted from fibroblast gel cultures at 6, 12, and 18 h of culture and was compared with mRNA of monolayer cultures. The transcription rate of the α_2 chain was highly increased after initiation of gel contraction (Fig. 5). mRNA levels of the β_1 chain were also elevated under the gel culture condition (not shown). The difference of the β_1 chain transcription rates, however, under monolayer and gel culture condition was significantly smaller than that of the α_2 chain.

To address the question of whether the selective induction of $\alpha_2\beta_1$ has functional relevance for gel contraction, we performed inhibition studies using function blocking mAb's directed against the α_2, α_3, α_5, and β_1 chain of integrins (see Table I). mAb W6/32 directed to HLA class I molecules was used as a control antibody. The mAbs were added to the gel culture when the fibroblasts were seeded into the gels.

MAbs 13 and 4B4 which are directed to the β_1 chain partially inhibited gel contraction (see also Table II). Titration experiments using antibody concentrations between 0.1 and 50 μg/ml revealed that the full inhibitory effect of each of the β_1 chain antibodies was already reached at concentrations of 0.6 μg/ml gel. When α_2, α_3, and α_5 chain mAbs or mAb W6/32 were alone added to the gels (concentrations ranging from 1 to 20 μg/ml), no inhibition of gel contraction was observed. Also, the combination of the α_2 chain mAb 5E8 with α_3 chain mAb P1B5 revealed no inhibitory effect.

We then studied the possibility of synergistic effects between β_1 and α chain mAbs. For this purpose, β_1 chain mAbs were used either at a suboptimal concentration of 0.4 μg/ml or at an optimal inhibitory concentration of 2.5 μg/ml and were combined with different α chain mAbs (2.5 μg/ml) or mAb W6/32 (2.5 μg/ml). The α_2 chain mAb 5E8 in conjunction with one of the anti-β_1 chain reagents further augmented the partial inhibition of gel contraction which was exerted by the β_1 chain mAbs alone. This synergistic effect could also be demonstrated when another α_2 chain mAb (P1E6) was used. The α_3 chain mAb P1B5, the α_5 chain mAb 16, however, and mAb W6/32 had no augmentory effect in combination with β_1 chain mAbs. A representative experiment is shown in Table II. From these experiments we

Table II. Influence of mAbs on Collagen I Gel Contraction by Adult Human Skin Fibroblasts

	Gel diameter (area)*		
mAb	18 h	24 h	48 h
0	8 (0,50)	7 (0,38)	7 (0,38)
HLA class I	8 (0,50)	7 (0,38)	7 (0,38)
α_5 chain	9 (0,64)	8 (0,50)	8 (0,50)
α_2 chain	8 (0,50)	7 (0,38)	7 (0,38)
β_1 chain (low conc)	10 (0,79)	9 (0,64)	0 (0,64)
β_1 chain plus HLA class I	10 (0,79)	10 (0,79)	10 (0,79)
β_1 chain plus α_5 chain	14 (1,54)	10 (0,79)	9 (0,64)
β_1 chain plus α_2 chain	25 (4,91)	16 (2,01)	15 (1,77)
β_1 chain (high conc)	27 (5,72)	15 (1,77)	15 (1,77)
β_1 chain plus HLA class I	23 (4,15)	15 (1,77)	15 (1,77)
β_1 chain plus α_5 chain	22 (3,80)	15 (1,77)	15 (1,77)
β_1 chain plus α_2 chain	29 (6,60)	20 (3,14)	20 (3,14)
α_2 chain plus α_5 chain	11 (0,95)	8 (0,50)	nd
α_2 chain plus HLA class I	10 (0,79)	8 (0,50)	nd

Experiments were performed in 35-mm Petri dishes. 4 × 10^5 fibroblasts (H-45) were seeded into 2 ml of gel containing 1.2 mg collagen I. The inner diameter of the dishes was 30 mm. Gel diameters were measured with a ruler. The area of an uncontracted collagen gel was 7.07 cm^2. Different mAbs were added to the gels during preparation. The mAb concentration was 2.5 μg/ml gel for all mAbs except the function blocking β_1 chain antibody 4B4, which was used either at a low concentration resulting in a marginal suboptimal inhibitory effect (0.4 μg/ml gel) or at a high concentration providing optimal inhibition (2.5 μg/ml gel). HLA class I mAb (W6/32); anti-α_5 chain mAb, which blocks function (mAb16); anti-α_2 chain mAb, which blocks function (5E8). nd, not done; 0, no mAb was added.
* gel diameter (area) in mm (cm^2).

*Table III. Contraction of Hydrated Collagen I Lattices by Human Melanoma Cells, Normal Melanocytes and Fibroblasts**

		48 h	96 h
Melanomas	MV3	12 (1,13)‡	10 (0,79)
	BLM	13 (1,33)	10 (0,79)
	530	n.c.	n.c.
	IF6	n.c.	n.c.
	Mewo	n.c.	34 (9,07)
	SK-Mel-13	40 (12,56)	33 (8,55)
	SK-Mel-19	n.c.	n.c.
	SK-Mel-29	n.c.	40 (12,56)
	SK-Mel-113	n.c.	n.c.
Melanocyte cultures	M.LM	n.c.	n.c.
	M.SD	n.c.	n.c.
	M.RF	n.c.	n.c.
	M.HF	n.c.	n.c.
Fibroblasts	H-45	12 (1,13)	10 (0,79)
	H-EK	10 (0,79)	10 (0,79)
	H-50	12 (1,13)	11 (0,95)
	H-63	9 (0,64)	9 (0,64)
	NH-1	8 (0,50)	8 (0,50)
	MU-2	8 (0,50)	8 (0,50)
	F135-60-86 skin	8 (0,50)	8 (0,50)

* Experiments were performed in 60-mm petri dishes (inner area 23.7 cm^2). *n.c.*, no contraction. 1.2×10^6 fibroblasts were seeded into 5 ml of gel containing 3-mg collagen I. The experiments were read after 48 and 96 h. Gel diameters were measured with a ruler.
‡ Gel diameter (area) in mm (cm^2).

concluded that the $\alpha_2\beta_1$ complex is of functional relevance for the gel contraction by fibroblasts.

To further study the involvement of $\alpha_2\beta_1$ in the reorganization of collagen I fibrils we chose another cell system and tested human melanoma cell lines and normal melanocyte cultures for their ability to contract collagen gels. This cell type appeared particularly interesting, since it has recently been found that $\alpha_2\beta_1$ is differentially expressed in normal and transformed melanocytic cells in vitro and is associated with tumor progression in vivo (Klein et al., 1991). Five out of nine melanoma cell lines were able to contract collagen I gels, whereas normal melanocytes were not (Table III). The contraction rate was significantly lower than that of diploid fibroblasts with the exception of the lines MV3 and BLM which contracted the gels with almost the same efficiency as fibroblasts. Two melanoma lines, MV3 and 530, were selected for further study and served as examples for contracting and noncontracting cells, respectively. First, we compared the synthesis of several integrins in monolayer culture. MV3 cells showed higher synthetic levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_4\beta_1$ than 530 (Fig. 6). $\alpha_5\beta_1$ and $\alpha_6\beta_1$ were expressed at very low and intermediate levels, respectively, in both lines (Fig. 6) whereas $\alpha_1\beta_1$ was not detectable (not shown). When seeded in collagen gels, MV3 cells revealed a significant induction of $\alpha_2\beta_1$ synthesis in comparison to the monolayer cultures (Fig. 6). Density scanning of the fluorographs showed a four- to eightfold increase. Other integrins were unchanged ($\alpha_5\beta_1$, $\alpha_v\beta_x$) or reduced ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$). The same results were obtained when the other "contracting" cell line BLM was studied. In contrast, upregulation of $\alpha_2\beta_1$ synthesis in gel culture was not seen in the "noncontracting" line 530 (Fig. 6).

To further link collagen gel contraction to $\alpha_2\beta_1$ function, we tried to block the contraction by MV3 cells with anti-$\alpha_2\beta_1$ mAbs. Again, function blocking mAbs directed to the α_2, α_3, and α_5 chain of integrins as well as the control mAb W6/32 were used alone or in combination with blocking mAbs directed to the β_1 chain. Here, it was found that the anti-α_2 chain mAb 5E8 alone inhibited gel contraction (Table IV and Fig. 7). However, complete inhibition was transient and the cells had partially overcome the inhibition after 48 h. Long lasting complete inhibition was not accomplished even at antibody concentrations of 50 μg/ml gel. Also, anti-β_1 chain mAbs alone (4B4 or 13) achieved full inhibition for a short time period only (Table IV). The anti-α_3 and α_5 chain mAbs P1B5 and 16, respectively, as well as control mAb W6/32 had no effect. However, when mAb 5E8 (anti-α_2) (2.5 μg/ml) was added to the gel culture in combination with mAbs 4B4 or 13 (anti-β_1) a long lasting complete inhibition of gel contraction was observed (Table IV and Fig. 7). The inhibition could not be overcome by the cells even after a culture period of 5 d without any further addition of mAbs. In normal diploid fibroblasts, complete inhibition had not been observed. Metabolic labeling of the "long-term inhibited" melanoma cultures with [^{35}S]methionine showed that the cells had retained their metabolic activity with protein synthesis levels as high as in 24 h gel cultures (not shown). The differential inhibitory effect of function blocking anti-α_2 and β_1 chain mAbs was confirmed in five experiments in which the influence of the mAbs on the contraction by fibroblasts and melanoma cells was studied in parallel.

Discussion

In this study, we investigated the involvement of integrin receptors in the reorganization of collagen I by human fibroblasts and melanoma cells. Among the three known collagen-binding integrins of the β_1 subfamily ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$) only $\alpha_2\beta_1$ was strongly upregulated in both cell types during gel contraction. The elevation in $\alpha_2\beta_1$ synthesis was closely associated with the contraction process and returned to baseline levels after contraction had subsided. Moreover, the induction of $\alpha_2\beta_1$ was also demonstrable in the transcriptional level.

Inhibition studies using mAbs which block ligand binding or function of β_1 integrins, revealed that the combination of anti-α_2 chain with anti-β_1 chain mAbs was most effective in inhibiting gel contraction. The comparison of the two cell types in respect to the inhibitory effect showed that these mAbs can block gel retraction of normal fibroblasts only partially whereas full- and long-lasting inhibition can be achieved in some melanoma cell lines (MV3 and BLM). This finding suggests that in fibroblasts, collagen receptors other than $\alpha_2\beta_1$ or collagen receptor independent mechanisms contribute to this process. For instance, the $\alpha_1\beta_1$ receptor which was synthesized in fibroblasts at low to intermediate levels could be involved. Since function blocking anti-α_1 chain mAbs were not available to us we could not exclude this possibility. In contrast to $\alpha_2\beta_1$, however, $\alpha_1\beta_1$ synthesis was not increased during gel contraction. The third collagen

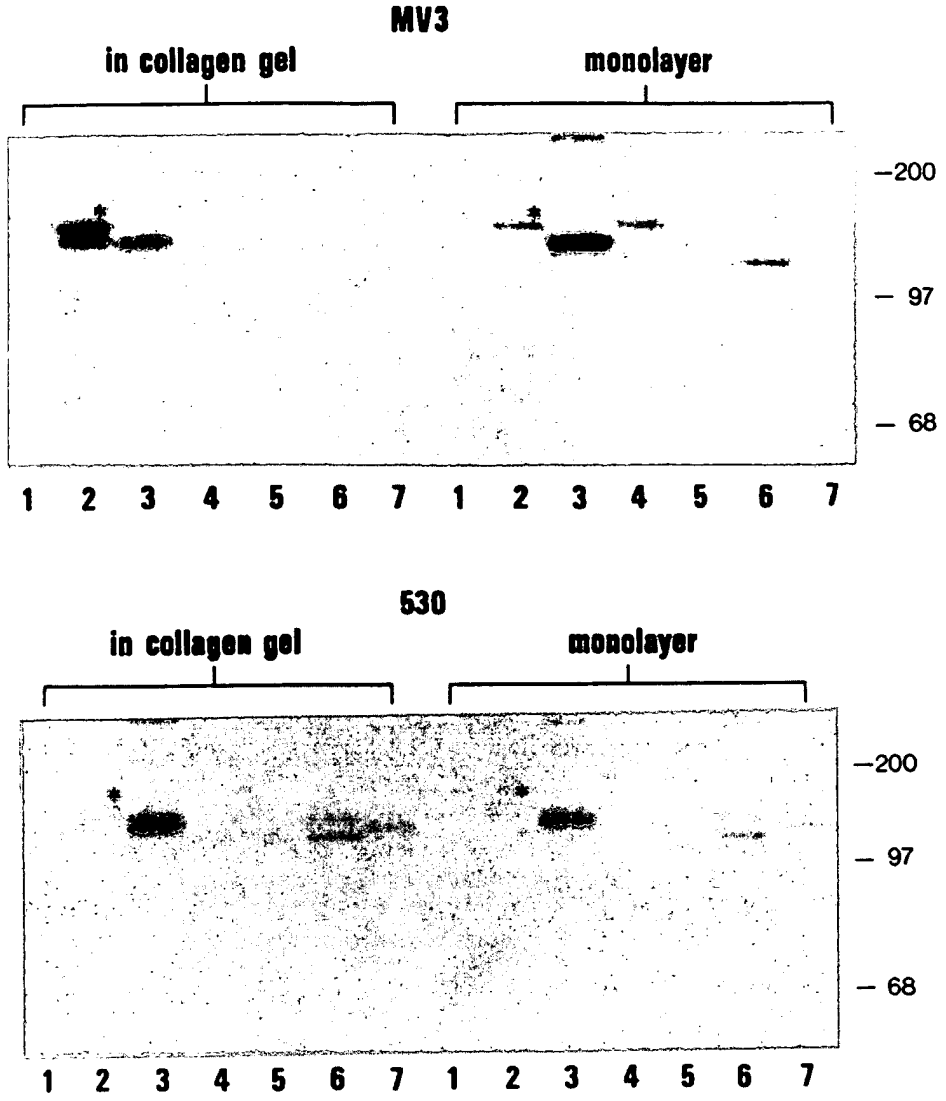


Figure 6. Synthesis of integrins in collagen I gel and monolayer cultures of two melanoma cell lines MV3 and 530. Con A–bound fractions of NP-40 cell lysates from [^{35}S]methionine-labeled parallel cultures were analyzed by immunoprecipitation and SDS-PAGE. The fluorographs show immunoprecipitates obtained with different mAbs: normal mouse serum (lane *1*); A-1-43, anti-α_2 (lane *2*); J143, anti-α_3 (lane *3*); P4G9, anti-α_4 (lane *4*); P1D6, anti-α_5 (lane *5*); GoH3 anti-α_6 (lane *6*); and LM142, anti-α_v (lane *7*). Asterisk indicate the $\alpha_2\beta_1$ immunoprecipitate. Note, that $\alpha_2\beta_1$ synthesis is strongly upregulated in gel cultures of MV3 cells which are able to contract the gels but not in 530 cells.

binding integrin $\alpha_3\beta_1$ appears not involved in gel contraction of fibroblasts. This is based on our observation that $\alpha_3\beta_1$ was synthesized at low levels and was not upregulated under gel culture conditions. More importantly, the anti-α_3 chain mAb P1B5 did not interfere with the contraction process.

In melanoma cells, gel contraction was completely blocked by the combination of anti-α_2 and anti-β_1 chain mAbs suggesting that $\alpha_2\beta_1$ is indispensable for the function in this cell type. In contrast to fibroblasts in which anti-α_2 chain mAbs alone had no inhibitory effect on gel contraction, the addition of anti-α_2 chain mAbs to melanoma cells resulted in partial inhibition of gel contraction. Full inhibition, however, could not be achieved with anti-α_2 chain mAbs alone even at high mAb concentrations. Interestingly, also anti-β_1 chain mAbs were alone incapable of a full- and long-lasting inhibitory effect on gel retraction by melanoma cells. This indicates that at least two extracellular domains, one on the α_2 and one on the β_1 chain are involved in this function. Since the binding of cells to collagen can efficiently be blocked by the anti-α_2 chain mAbs used (5E8, Bankert, unpublished results, and P1E6, Wayner et al., 1988), the requirement of a second epitope on the β_1 chain for full inhibition possibly indicates that the prevention of collagen binding to $\alpha_2\beta_1$ is not the only crucial aspect of integrin function in this process.

The view that $\alpha_2\beta_1$ is the collagen-binding integrin primarily involved in collagen gel reorganization by melanoma cells is further underscored by our observations: (*a*) that $\alpha_1\beta_1$ is not synthesized by the melanoma lines which were used in gel contraction assays; (*b*) that the synthetic levels of $\alpha_3\beta_1$ are reduced during gel culture; (*c*) and that the function-blocking anti-α_3 chain mAb P1B5 has no inhibitory effect.

Very recently, evidence has been provided that cellular fibronectin (cFN) has an important role for the reorganization of collagen I gels by fibroblasts (Asaga et al., 1991). It was suggested that cFN on the cell surface mediates gel contraction by binding to collagen I via its collagen binding domain. No evidence, however, was provided, which cellular receptor binds cFN under this condition. Possible candidates are the "classical" fibronectin receptor $\alpha_5\beta_1$, the integrin

Table IV. Influence of mAb on Collagen I Gel Contraction by Human Melanoma Cells

mAb	Gel diameter (area)*		
	18 h	24 h	48 h
0	21 (3.46)	13 (1.33)	10 (0.79)
HLA class I	24 (4.52)	12 (1.13)	10 (0.79)
α_5 chain	25 (4.91)	13 (1.33)	10 (0.79)
α_2 chain	30 (7.07)	30 (7.07)	15 (1.77)
β_1 chain (low conc.)	30 (7.07)	20 (3.14)	10 (0.79)
β_1 chain plus HLA class I	28 (6.15)	20 (3.14)	10 (0.79)
β_1 chain plus α_5 chain	27 (5.72)	18 (2.54)	10 (0.79)
β_1 chain plus α_2 chain	30 (7.07)	30 (7.07)	30 (7.07)
β_1 chain (high conc.)	30 (7.07)	20 (3.14)	12 (1.13)
β_1 chain plus HLA class I	30 (7.07)	27 (5.72)	12 (1.13)
β_1 chain plus α_5 chain	30 (7.07)	26 (5.31)	12 (1.13)
β_1 chain plus α_2 chain	30 (7.07)	30 (7.07)	30 (7.07)

Experiments were performed in 35-nm Petri dishes. 4×10^5 MV3 melanoma cells were seeded into 2 ml of gel containing 1.2 mg collagen I. The inner diameter of the dishes was 30 mm. Gel diameters were measured with a ruler. The area of an unconcentrated collagen gel was 7.07 cm^2. Different mAbs were added to the gels during preparation. The mAb concentration was 2.5 μg/ml gel for all mAbs except the function blocking β_1 chain mAb 4B4, which was used at a low concentration resulting in marginal inhibition (0.4 μg/ml gel) or at the high concentration providing optimal inhibition (2.5 μg/ml gel). HLA class I mAb (W6/32); α_5 chain mAb, which blocks function (mAb16); α_2 chain mAb, which blocks function (5E8). *0*, no mAb was added.

* Gel diameter (area) in mm (cm^2).

$\alpha_4\beta_1$ which binds to the CS-1 region of fibronectin (Wayner et al., 1989), and the "multifunctional" receptor $\alpha_3\beta_1$. An involvement of $\alpha_4\beta_1$ is unlikely, because it was clearly downregulated during gel contraction. Also $\alpha_5\beta_1$ and $\alpha_3\beta_1$ are probably not involved since mAbs interfering with α_5 and α_3 chain function showed no inhibitory effect on gel contraction. Because interference with $\alpha_2\beta_1$ led to partial inhibition of collagen gel contraction and because there is no direct evidence for the involvement of other collagen receptors, one has to take into consideration that collagen receptor independent mechanisms mediated by cFN take a substantial part in the reorganization of collagen I by fibroblasts.

The contraction of collagen gels by cultured fibroblasts is considered to reflect an important aspect of the wound healing process. Our finding that the human $\alpha_2\beta_1$ is upregulated during this process now directs attention to a possible role for wound healing in vivo. Here, immunohistological studies of healing wounds will be helpful in addressing this issue. Moreover, the availability of function blocking mAbs directed to the α_2 and β_1 chains of mouse integrins or other mammalian species will enable scientists to analyze whether the antibodies interfere with wound healing processes in animal models.

Recent studies of α_5 and β_1 chain expression in healing porcine wounds led to the observation that the fibronectin receptor $\alpha_5\beta_1$ is expressed by fibroblasts during an intermediate stage of wound healing just before wound contraction (Welch et al., 1989; Clark, 1990). Based on these findings, the concept was put forward that $\alpha_5\beta_1$ is functionally relevant for wound contraction in vivo. The results of the study presented here suggest that—at least under in vitro conditions in which collagen I is the only extracellular matrix protein provided—$\alpha_5\beta_1$ function is irrelevant for collagen gel contraction. This is based on the observations that anti-α_5 chain mAbs did not influence the gel contraction rate of fibroblasts and that the synthetic levels of $\alpha_5\beta_1$ were unchanged.

In the second part of this study we investigated the ability of melanoma cell lines to contract collagen gels and the role of $\alpha_2\beta_1$ in respect to this process. Five out of nine melanoma cell lines contracted collagen gels. Most of these were relatively ineffective in contracting gels when compared to normal fetal or adult fibroblasts. However, two melanoma cell lines (MV3 and BLM) demonstrated contraction efficiencies comparable to that of fibroblasts whereas normal melanocytes were not able to reorganize gels. These two lines were recently established in an effort to obtain human melanoma cells, which are highly aggressive after transplantation in immunocompromised mice. Both cell lines generated metastases at high frequency in nu/nu mice after subcutaneous inoculation (van Muijen et al., 1989, 1991). We have found that these cells strongly upregulate $\alpha_2\beta_1$ when seeded into collagen I gels. The view that the upregulation of $\alpha_2\beta_1$ is a critical prerequisite for gel contraction is further supported by our observation that the melanoma line 530 which was not capable to contract gels, did not increase $\alpha_2\beta_1$ synthesis in gel culture. Interestingly, 530 was also not capable of forming lung metastases in nu/nu mice (van Muijen, personal communication). These observations point to an interesting association of the ability of melanoma cells to contract collagen I gels with their metastatic potential. The link between the two phenomena possibly represents the ability to upregulate $\alpha_2\beta_1$ expression. Furthermore, it is likely, that the ability of remodelling connective tissue in itself provides substantial advantages for melanoma cells during tumor progression.

Several recent observations strengthen the concept that increased expression of $\alpha_2\beta_1$ is associated with malignant transformation and that $\alpha_2\beta_1$-mediated functions favor tumor progression. We have recently demonstrated that a previously characterized tumor progression antigen, which is expressed at increased frequency in primary melanomas and melanoma metastases relative to benign melanocytic lesions is identical to the integrin $\alpha_2\beta_1$ (Klein et al., 1991). F. A. Chen et al. (1991) found that human lung tumors (non-small cell lung cancer) express at least twenty times more integrin α_2 chain message than normal adult lung tissue. Furthermore, Yamada et al. (1990) reported that mAbs directed to the α_2 chain of integrins strongly inhibited migration of tumor cells in three-dimensional collagen gels. Moreover, transfection and overexpression of the human $\alpha_2\beta_1$ integrin leads to an increased metastatic potential of the recipient cells (Chan et al., 1991).

The results of our present study further support the assumption that the ability to express and upregulate $\alpha_2\beta_1$ is an important feature in the reorganization of the connective tissue during wound healing but also plays a critical role for tissue invasion and metastasis of tumor cells.

We thank Hilde Heim for expert secretarial assistance.

This work was supported in part by grants from the German Research Foundation DFG KL 510/2-2 to C. E. Klein and DFG Kr 558/6-1 to T. Krieg.

Received for publication 15 April 1991 and in revised form 19 July 1991.

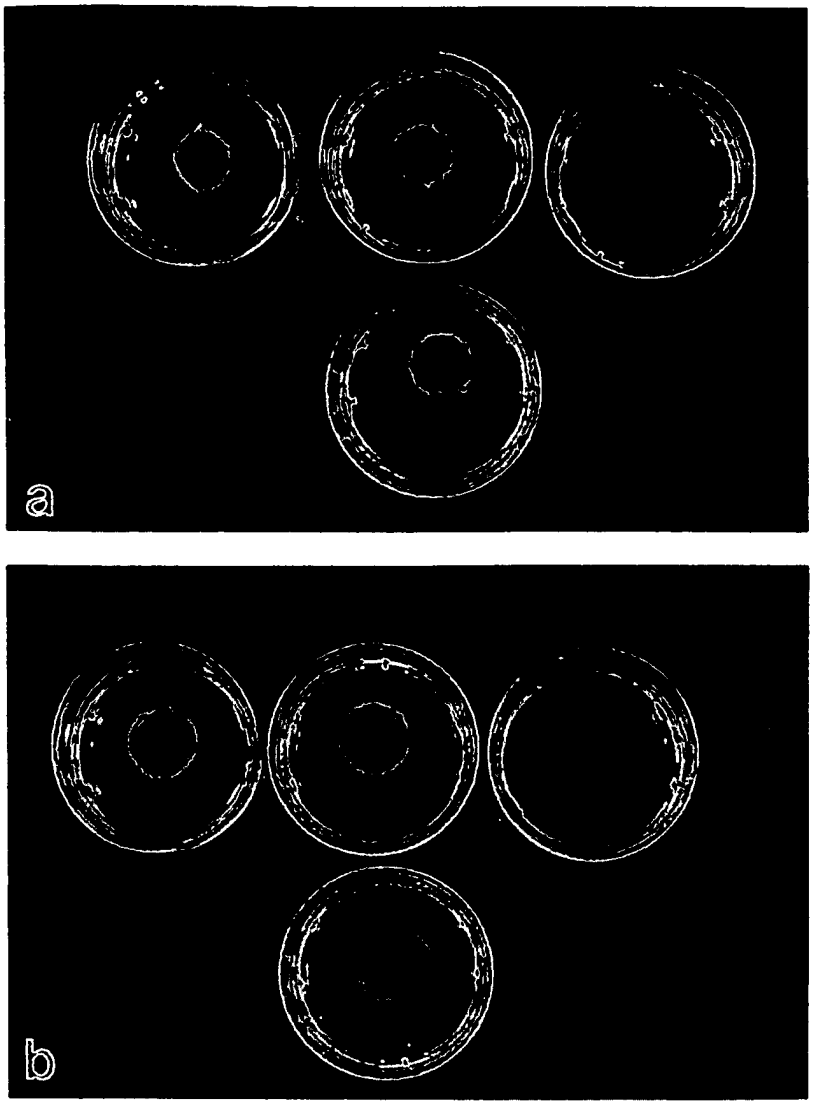


Figure 7. Inhibition of collagen I gel contraction by MV3 melanoma cells by anti-α_2 and β_1 chain mAbs. Collagen I gel cultures (2 ml) containing 4×10^5 MV3 melanoma cells were prepared in 35-mm petri dishes. mAbs were added during preparation of the gels. Photographs were taken after 30 h of culture. (*A*) The following mAbs (2.5 μg/ml) were added to the cultures. (Upper row, from left to right): W6/32 (anti-HLA class I); 16 (anti-α_5 chain); 5E8 (anti-α_2 chain). (Lower row, single dish): no antibody. (*B*) Each dish contained 0.04 μg/ml mAb 4B4 (anti-β_1 chain). To study synergistic effects with 4B4, the following mAbs (2.5 μg/ml) had been added: (Upper row from left to right): W6/32 (anti-HLA class I); 16 (anti-α_5 chain); 5E8 (anti-α_2 chain). (Lower row, single dish): mAb 4B4 only.

References

Akyama, S. K., S. S. Yamada, W. T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly and cytoskeletal organization. *J. Cell Biol.* 109:863–875.

Argraves, W. S., S. Suzuki, H. Avai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.

Asaga, H., S. Kikuchi, and K. Yoshizato. 1991. Collagen gel contraction by fibroblasts requires cellular fibronectin but not plasma fibronectin. *Exp. Cell Res.* 193:167–174.

Belkin, V. M., A. M. Belkin, and V. E. Koteliansky. 1990. Human smooth muscle VLA-1 integrin: purification, substrate specificity, localization in aorta, and expression during development. *J. Cell Biol.* 111:2159–2170.

Bell, E., B. Ivarsson, and C. Merill. 1979. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc. Natl. Acad. Sci. USA.* 76:1274–1278.

Chan, B. M. C., N. Matsuura, Y. Takada, B. R. Zetter, and M. E. Hemler. 1991. In vitro and in vivo consequences of VLA-2 expression on rhabdomyosarcoma cells. *Science (Wash. DC).* 251:1600–1602.

Chen, F. A., E. A. Repasky, and R. B. Bankert. 1991. Human lung tumor associated antigen identified as an extracellular matrix adhesion molecule. *J. Exp. Med.* 173:1111–1119.

Cheresh, D. A., and R. C. Spiro. 1987. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand Factor. *J. Biol. Chem.* 262:17703–17711.

Clark, R. A. F. 1990. Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. *J. Invest. Dermatol.* 94:128S–134S.

Dedhar, S., and R. Saulnier. 1990. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J. Cell Biol.* 110:481–489.

Eisinger, M., and O. Marko. 1982. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. *Proc. Natl. Acad. Sci. USA.* 79:2018–2022.

Guidry, C., and F. Grinell. 1985. Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts. *J. Cell Sci.* 79:67–81.

Gullberg, D., A. Tingström, C. Thuresson, L. Olsson, L. Terracio, T. K. Borg, and K. Rubin. 1990. β_1 Integrin-mediated collagen gel contraction is stimulated by PDGF. *Exp. Cell Res.* 186:264–272.

Halaban, R., S. Gosh, P. Duray, J. M. Kirkwood, and A. B. Lerner. 1986. Human melanocytes cultured from nevi and melanomas. *J. Invest. Dermatol.* 87:95–101.

Hemler, M. E., J. G. Jacobson, and J. L. Strominger. 1985. Biochemical characterization of VLA-1 and VLA-2 cell surface heterodimers on activated T cells. *J. Biol. Chem.* 260:15246–15252.

Hemler, M. E., C. Huang, and Y. Takada. 1987. Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* 262:11478–11485.

Hemler, M. E., C. Crouse, Y. Takada, and A. Sonnenberg. 1988. Multiple very late antigen (VLA) heterodimers on platelets. Evidence for distinct VLA-2, VLA-5 (fibronectin receptor) and VLA-6 structures. *J. Biol. Chem.* 263:7660–7665.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

Kantor, R. R. S., M. J. Mattes, and K. O. Lloyd. 1987. Biochemical analysis of two cell surface glycoprotein complexes, very common antigen 1 and very

common antigen 2. Relationship to very late activation T cell antigens. *J. Biol. Chem.* 262:15158–15165.

Kaufman, R., D. Frösch, C. Westphal, L. Weber, and C. E. Klein. 1989. Integrin VLA-3: Ultrastructural localization at cell-cell contact sites of human cell cultures. *J. Cell Biol.* 109:1807–1815.

Kirchhofer, D., L. R. Languino, E. Ruoslahti, and M. D. Pierschbacher. 1990. $\alpha_2\beta_1$-integrins from different cell types show different binding specificities. *J. Biol. Chem.* 265:615–618.

Klein, C. E. 1988. A transformation associated 130 kD cell surface glycoprotein is growth controlled in normal human cells. *J. Exp. Med.* 167:1684–1696.

Klein, C. E., T. Steinmayer, J. Mattes, R. Kaufmann, and L. Weber. 1990. Integrins of normal human epidermis: differential expression, synthesis and molecular structure. *Br. J. Dermatol.* 123:171–178.

Klein, C. E., T. Steinmayer, D. Kaufmann, and L. Weber. 1991. Identification of a melanoma progression antigen as integrin VLA-2. *J. Invest. Dermatol.* 96:281–284.

Kramer, R. H., K. A. McDonald, and M. P. Vu. 1989. Human melanoma cells express a novel integrin receptor for laminin. *J. Biol. Chem.* 264:15642–15649.

Lloyd, K. O., J. Ng, and W. G. Dippold. 1981. Analysis of the biosynthesis of HLA-DR glycoprotein in human malignant melanoma cell lines. *J. Immunol.* 126:2408–2413.

Mauch, C. 1986. Ph.D. thesis. Zell-Matrix-Wechselwirkungen von Fibroblasten. Physiologische und pathophysiologische Aspekte. Naturwissenschaftliche Fakultät III der Universität Regensburg, Regensburg, Germany.

Mauch, C., A. Hatamochi, K. Scharfetter, and T. Krieg. 1988. Regulation of collagen synthesis in fibroblasts within a three dimensional collagen gel. *Exp. Cell Res.* 178:493–503.

Morimoto, C., N. L. Letvin, J. A. Distaso, W. R. Aldrich, and S. F. Schlossman. 1985. The isolation and characterization of the human suppressor inducer T Cell subset. *J. Immunol.* 134:1508–1515.

Muijen van, G. N. P., I. M. H. A. Cornelissen, C. F. J. Jansen, and D. J. Ruiter. 1989. Progression markers in metastasizing human melanoma cells xenografted to nude mice. *Anticancer Res.* 9:879–884.

Muijen van, G. N. P., C. F. J. Jansen, L. M. H. A. Cornelissen, J. L. M. Beck, and D. J. Ruiter. 1991. Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. *Int. J. Cancer.* 48:85–91.

Nakagawa, S., P. Pawelek, and F. Grinnell. 1989. Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. *Exp. Cell Res.* 182:572–582.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RDG and integrins. *Science (Wash. DC).* 238:491–497.

Shimizu, Y., G. A. van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA (β_1) integrin receptors on T cells. *Nature (Lond.).* 345:250–253.

Santoso, S., V. Kiefel, and C. Müller-Eckhardt. 1989. Immunochemical characterization of the new platelet alloantigen system Br^a/Br^b. *Br. J. Haematol.* 72:191–198.

Sonnenberg, A., P. W. Modderman, and F. Hogervorst. 1988. Laminin receptor on platelets is the integrin VLA-6. *Nature (Lond.).* 336:487–489.

Takada, Y., and M. E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor α_2-subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109:397–407.

Tiollier, J., H. Dumas, M. Tardy, and J.-L. Tayot. 1990. Fibroblast behavior on gels of type I, II and IV human placental collagens. *Exp. Cell Res.* 191: 95–104.

Tomasek, J. J., E. D. Hay, and K. Fujiwara. 1982. Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts: Distribution of actin, α-Actinin and myosin. *Dev. Biol.* 92:107–122.

Versteeg, R., I. O. A. Noordermeer, M. Krüse-Wolters, D. J. Ruiter, and P. I. Schrier. 1988. C-myc down-regulates class I HLA expression in human melanomas. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1023–1029.

Wayner, E. A., and W. G. Carter. 1987. Identification of multiple cell adhesion receptors for type VI collagen and fibronectin in human fibrosarcoma cells possessing unique α and common β-subunits. *J. Cell Biol.* 105:1873–1884.

Wayner, E. A., W. G. Carter, R. S. Pistrowicz, and T. J. Kunicki. 1988. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.* 107:1881–1891.

Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T-lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.

Welch, M. P., G. F. Odland, and A. F. Clark. 1990. Temporal relationships of F-Actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. *J. Cell Biol.* 110: 133–145.

Yamada, K. M., D. W. Kennedy, S. S. Yamada, H. Gralnick, W. T. Chen, and S. K. Akiyama. 1990. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res.* 50:4485–4496.

Zylstra, S., F. A. Chen, S. K. Ghosh, E.A. Repasky, U. Rao, H. Takita, and R. B. Bankert. 1986. Membrane-associated glycoprotein (gp 160) identified on human lung tumors by a monoclonal antibody. *Cancer Res.* 46:6446–6451.

DEVELOPMENTAL BIOLOGY 175, 301-313 (1996)
ARTICLE NO. 0116

Deletion of Integrin $\alpha 1$ by Homologous Recombination Permits Normal Murine Development but Gives Rise to a Specific Deficit in Cell Adhesion

Humphrey Gardner,*,[1] Jordan Kreidberg,†
Victor Koteliansky,‡ and Rudolf Jaenisch*

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142; †Children's Hospital, Boston, Massachusetts 02115; and ‡Section de Recherche, Institut Curie, UMR 144 du CNRS Compartimentation et dynamique cellulaires, 75231 Paris Cedex, France

Integrin $\alpha 1$ is a receptor for laminin and collagen which is expressed widely and dynamically in embryogenesis and has been implicated in various developmental processes including establishment of the placenta and formation of the central and peripheral nervous system. In the adult it is the sole collagen receptor in smooth muscle and liver and is thought to be important for the stability of these tissues. We have generated a null allele of the $\alpha 1$ gene in the germline of mice by homologous recombination in embryonic stem cells. Mice homozygous for the mutation are viable and fertile and have no overt phenotype, demonstrating that the molecule is not required for development. Embryonic fibroblasts derived from mutant animals are unable to spread on or migrate into substrata of collagen IV and are deficient in spreading on and migrating into laminin. Further *in vitro* analysis of cell spreading and migration suggests that $\alpha 1\beta 1$ is not required for binding to collagen I and implicates a third receptor, possibly integrin $\alpha 3\beta 1$, in collagen I binding.

INTRODUCTION

The integrins are a group of heterodimeric transmembrane proteins responsible for binding extracellular matrix ligands as well as communicating signals from the matrix to the cytoskeleton and various intracellular signal transduction pathways (Hynes, 1992; Ruoslahti, 1991). An integrin heterodimer consists of an α and a β subunit. In mammals there are at least 8 β and 14 α subunits, and their various combinations give rise to a great variety of heterodimers, which differ both in their ligand specificity and in the signals they can transduce to the cytoplasm. In general, it is the α subunit which dictates the ligand specificity of an integrin while the β subunit cytoplasmic terminus interacts with the cytoskeleton and with proteins involved in signal transduction. Integrins have a central role in the maintenance of the integrity of the organism. They control cell shape and interaction with extracellular proteins, confer anchorage dependence for cell survival and division, and are crucial for events of cell migration in development, repair, and other physiological necessities such as blood clotting. The $\beta 1$ family of integrins supplies most of the mechanical attachment to extracellular matrix molecules, and absence of the $\beta 1$ subunit is lethal to embryos prior to implantation (Fassler and Meyer, 1995). Various individual members of the $\beta 1$ family have been shown to be of importance in embryonic development, by mutation of either the α subunits or their ligands. Targeted deletions of $\alpha 4$, $\alpha 5$, and of one of their ligands, fibronectin (George *et al.*, 1993; Yang *et al.*, 1993, 1995), have caused embryonic death. While targeted deletions of the collagen- and laminin-binding heterodimers have not been reported hitherto, the importance of their ligands has been emphasized by genetic analysis. Ablation of transcription of the collagen I gene col1A1 by retroviral

[1] To whom correspondence should be addressed at The Scripps Research Institute, 10666 North Torrey Pines Road, Mail Drop CAL3, La Jolla, CA 92037. Fax: (619) 554-6945. E-mail: humphrey@scripps.edu.

0012-1606/96 $18.00

insertion is lethal to mouse embryos around Day 13 (Schnieke *et al.*, 1983) and some collagen IV mutations are embryonic lethals in *Caenorhabditis elegans* (Olsen, 1995). Targeted deletion of S-Laminin (Noakes *et al.*, 1995) causes glomerulonephritis and aberrant neuromuscular junction formation, while a naturally occurring mouse mutant lacking Merosin develops muscular dystrophy (Xu *et al.*, 1994).

Integrin $\alpha 1$ was first identified as the largest of a series of very late antigens (VLAs) expressed *in vitro* by T cells (Hemler *et al.*, 1984, 1987). It has been cloned in rat (Ignatius *et al.*, 1990) and human (Briesewitz *et al.*, 1993) and the two proteins show a greater than 90% identity. The $\alpha 1$ chain is 1152 residues long, with a 15-amino-acid cytoplasmic C-terminus. In common with integrin $\alpha 2$, and the α partners of the $\beta 2$ integrins, it has an extracellular I domain, which has been demonstrated to be crucial for binding activity (Kern *et al.*, 1994). Integrin $\alpha 1$ has only one known partner, $\beta 1$, and the $\alpha 1\beta 1$ heterodimer is a receptor for collagens and laminins (Belkin *et al.*, 1990; Colognato-Pyke *et al.*, 1995; Kramer and Marks, 1989; Kuhn and Eble, 1994; Ignatius and Reichardt, 1988; Tawil *et al.*, 1990). On collagen I $\alpha 1\beta 1$ has two sites of attachment, one in the CB8 fragment and the other in CB3 (Gullberg *et al.*, 1992) while on laminin the binding site is in domain VI of the short arm of the α chain (Colognato-Pyke *et al.*, 1995). The site of $\alpha 1\beta 1$ binding on collagen IV is striking in that it consists of three residues, each of which is on a different chain of the triple helical portion of the molecule, namely Asp 461 of both α chains and Arg 461 of the β chain (Kuhn and Eble, 1994), which may account for the lack of small peptides known to inhibit $\alpha 1$ function.

Expression of $\alpha 1$ in the adult animal is predominantly mesenchymal and quite distinct from the predominantly epithelial collagen receptor $\alpha 2$ (Wu and Santoro, 1994). $\alpha 1$ is found in visceral and some vascular smooth muscle (Belkin *et al.*, 1990; Glukhova *et al.*, 1993), the liver (Gullberg *et al.*, 1990), microvascular endothelium (Defilippi *et al.*, 1991), glomerular mesangium (Korhonen *et al.*, 1990a,b), the myoepithelium of the mammary gland (Glukhova *et al.*, 1995) chondrocytes (Loeser *et al.*, 1995), and is absent from epithelia. During development, $\alpha 1$ has a very dynamic pattern of expression. It is first seen at the leading edge of invading trophoblast shortly after implantation (Sutherland *et al.*, 1993), and antibody blockade of $\alpha 1$ inhibits trophoblast invasion *in vitro* (Damsky *et al.*, 1994). During early to mid embryogenesis $\alpha 1$ is expressed transiently by neurons of the CNS (Duband *et al.*, 1992), by maturing skeletal and cardiac muscle (Terracio *et al.*, 1991), in the skin (Hertle *et al.*, 1991), throughout the developing kidney (Korhonen *et al.*, 1990a), and in neural crest cells as they mature to dorsal root ganglia (Duband *et al.*, 1992). *In vitro,* it has been found to be important for neurite outgrowth on laminin (Tomaselli *et al.*, 1993) and neural crest cell attachment to collagen (Perris *et al.*, 1993). Neural crest cell attachment to laminin can be inhibited by antisense oligonucleotides to $\alpha 1$ mRNA (Lallier and Bronner-Fraser, 1993).

Various growth factors have been shown to regulate $\alpha 1$ expression, including NGF (Rossino *et al.*, 1990), TGFβ (Serra *et al.*, 1994), PDGF (Janat *et al.*, 1992), and TNF (Defilippi *et al.*, 1991). Conversely, TNF secretion in activated T cells can be induced by collagen binding via $\alpha 1$ (Miyake *et al.*, 1994). Expression of $\alpha 1$ integrin is also altered in various disease states. Macrovascular endothelium, such as that of the aorta, usually does not express $\alpha 1$, but does do so after injury (Defilippi *et al.*, 1991). $\alpha 1$ is absent from lymphocytes of the peripheral blood; it is, however, produced by CD8 cells in the synovium of patients afflicted with rheumatoid arthritis (Hemler *et al.*, 1986; Hemler and Jacobson, 1987) and upregulated in intraepithelial and lamina propria gut lymphocytes in graft versus host disease (Tanaka *et al.*, 1995). $\alpha 1$ is upregulated in the chondrocytes of osteoarthritics (Loeser *et al.*, 1995). In contrast to $\alpha 2$, $\alpha 1$ is not seen in normal skin or after cutaneous injury (Juhasz *et al.*, 1993). Dysregulation of $\alpha 1$ has also been reported in various cancers. The molecule is upregulated in a proportion of bladder carcinomas (Liebert *et al.*, 1994) and in the malignant lymphocytes of the epidermotrophic lymphoma mycosis fungoides (Sterry *et al.*, 1992). Downregulation of integrin $\alpha 1$ has been described in the smooth muscle cells of leiomyosarcomata (Mechtersheimer *et al.*, 1994) and is also a common finding in small cell cancer of the lung (Suzuki *et al.*, 1993), adenocarcinoma of the lung (Roussel *et al.*, 1994), and nodal metastatic breast cancer (Gui *et al.*, 1995). Furthermore, TGFβ suppresses the transformed phenotype of ras transformed hepatocytes by causing an upregulation of $\alpha 1$ (Serra *et al.*, 1994).

This evidence suggests that integrin $\alpha 1\beta 1$ may have many different functions. In development its importance might be as an aid to cellular migration or even in the induction of a differentiated state when cells have found the appropriate extracellular milieu. In the adult it might be expected to provide structural integrity to various tissues, particularly liver and smooth muscle, where it is the sole collagen receptor. In disease, $\alpha 1\beta 1$ is dysregulated in cells which have in common a requirement for migration. It was therefore a promising target for deletion by homologous recombination, as the first step in dissection of cellular interactions with laminin and collagen *in vivo*. In this paper we describe the phenotype of $\alpha 1$ deficient mice.

MATERIALS AND METHODS

Cloning of Mouse $\alpha 1$ Genomic Sequence and Preparation of the Targeting Construct

A 1.8-kb human integrin $\alpha 1$ cDNA (gift of E. Marcantonio) (Briesewitz *et al.*, 1993) was used to probe a mouse cDNA library. Three overlapping cDNA clones were isolated, sequenced, and found to extend 800 bp from the 5′ UTR to the I domain of integrin $\alpha 1$. They showed greater than 95% homology to the rat α-1 sequence (Ignatius *et al.*, 1990). The 5′ cDNA was used to isolate a 9-kb genomic fragment from a 129/sv genomic library. The latter

was mapped and partial sequencing revealed two exons near the N terminus, the 5′ one of which had a unique *ClaI* site. Fragments of the genomic clone were inserted to flank a pgkneo polyA cassette (Rudnicki *et al.*, 1992) to give the plasmid pNCNeoCT such that the neo cassette was inserted at the *ClaI* site of the genomic sequence, in reverse orientation (Fig. 1A, plasmid). This plasmid was cleaved with *SacI* and *XhoI* and ligated in a three-part reaction with the large *Xho/Hin*dIII fragment of pMC1TKpolyA (to supply a TK gene for counterselection (Mansour *et al.*, 1988)) and pUC19 cleaved with *SacI* and *Hin*dIII, to give the transfection construct pJKa1.

Transfection of ES Cells

Two independent transfections of 7×10^7 J1 cells were made. Each transfection used 25 μg pJKa1 linearized at the unique *XhoI* site. Transfections were performed by electroporation using a Bio-Rad Gene Pulser with a 0.4-cm cuvette at 250 V, 25 μF, in a volume of 0.7 ml, in electroporation buffer (Li *et al.*, 1992). These conditions gave a time constant of 4–5. Each transfection was immediately plated onto five 10-cm dishes of neo-resistant irradiated embryonic fibroblasts in ES medium (Li *et al.*, 1992) supplemented with 500 U/ml LIF (Gibco). Selection consisting of 170 μg/ml G418 + 0.2 μM FIAU was added at 24 hr. Selective medium was changed daily. One dish from each electroporation was selected with G418 only. Colonies were picked at 9 or 10 days, and expanded in 24-well plates in the same medium. When wells neared confluence, they were trypsinised and half the cells were frozen in 10% FCS, 10% DMSO. The remaining cells were expanded to confluence and used for DNA preparation (Laird *et al.*, 1991).

Chimeras

Homologously targeted recombinant clones were thawed and expanded in nonselective ES medium with 500 U/ml LIF. Cells were injected into E3.5 BALB/C blastocysts at 10–15 cells per injection. Embryos were transferred into E 2.5 pseudopregnant Swiss Webster fosters, at 8–10 blastocysts per uterine horn. Coat color chimeras were mated to BALB/C dams.

Genotyping of Offspring

Tail DNA was prepared as according to Laird *et al.* (1991). Tail DNA was subjected to PCR in 20 μl reaction volume with 1.25 mM $MgCl_2$ and 1 pmole/μl each primer using a mix of three primers. Primer sequences were as follows: w (wild type) 5′ gttgttctatttttgtagttaac 3′; k (knockout) 5′ ggggaacttcctgactag 3′; and c (common) 5′ aatcctccattcgggttggtg 3′. DNA was initially denatured for 3 min at 95°C, followed by 32 cycles of: 95°C, 30s, 55°C, 1 min, 72°C, 2 min. The reaction was run on 1.5% agarose and gave a 103-bp wild-type band and/or a 273-bp ko band.

Isolation of Cells from Embryonic and Adult Tissue

Embryonic fibroblasts (EFs) were obtained from E14.5 embryos according to the method of Li *et al.* (1992). Smooth muscle cells (SMCs) were obtained from adult uteri by the method of Erulkar *et al.* (1994). EFs were transformed by infection with SV40 containing retrovirus as described by Mann *et al.* (1983).

Cell Spreading Studies

Cell spreading assays were performed essentially as described (Gardner *et al.*, 1994). Briefly, 24-well plates were coated with 0.5 ml/well collagen I or IV (Sigma) or laminin (EHS laminin, Gibco) at the indicated concentrations in PBS+, or with FCS, for 4 hr at 37°C and subsequently blocked with 10% BSA (Fraction V, Sigma) for 2–12 hr before plating. Cells were washed twice in DME/0.1% BSA and plated at 50–100,000 per well in the same medium. When antibodies were used, 1/10 volume of antibody was added as hybridoma culture supernatant (anti-α1, α2, and β1, hamster anti-rat, gifts of Donna Mendrick) and the cells were incubated on ice for 30 min prior to plating. At 1 hr, two random fields at 200× magnification near the center of the well were counted. Round, refractile cells were counted as unspread, while flatter cells with cytoplasmic extensions were counted as spread. Spread cells in each field were expressed as a percentage of the total. Results are expressed as the average percentage of spread cells in the two independent wells, while error bars indicate the higher value.

Cell Migration Assay

Migration assays were performed as described (Pasqualini and Hemler, 1994). Briefly, modified Boyden chambers with 8-μm membrane perforations (Costar 3422) were coated on the underside with 40 μl of collagen I, collagen IV, or laminin at the indicated concentrations in PBS+, or with FCS, and incubated for 1 hr at room temperature under laminar flow. They were blocked with 10% BSA in PBS+ under the same conditions, and then rinsed in the well with DME/0.1% BSA. They were transferred to new wells containing 600 μl of the same medium. Cells (50,000) were added to the upper chamber in a 100-μl volume of DME/0.1% BSA/2% FCS, and the plate was incubated at 37°C for 5 hr. When antibodies were used, 1/10 volume of antibody was added to the cells 30 min before addition to the well. Inserts were removed, fixed in methanol for 5 min, and stained with Giemsa for 5 min, and the membranes were detached and placed on glass slides, underside down. The upper side of the membrane was wiped free of cells, and cells on the underside were counted at 10× objective magnification. Four random fields were counted for each membrane. Results are expressed as the mean of two independent experiments, with the error bar indicating the higher value.

Immunoprecipitation

Cells were trypsinised, washed, and labeled with NHS LC biotin (Pierce), according to manufacturer's instructions. Cell lysates and immunoprecipitations were prepared as according to Yang *et al.* (1993). Lysates were separated on 6% SDS acrylamide gels and transferred to nitrocellulose (Amersham). Biotinylated proteins were visualized with the Vectastain elite ABC kit (Vector Labs) and Amersham ECL reagents (Amersham) according to manufacturers' instructions The anti-β-1 antibody K363 was a gift of Joy Yang. Anti-cytoplasmic peptide antisera to integrins α1, α2, and α3 were a gift of Guido Tarone.

Immunohistochemistry

Immunohistochemistry was performed on air-dried acetone-fixed 7-μm frozen sections using hamster anti-rat monoclonal anti-α1 and anti-α2 antibodies (a gift of Donna Mendrick), or polyclonal rabbit anti-mouse α2 (gift of S. Santoro). Sections were preincubated in 3% BSA/PBS (monoclonals) or 10% goat serum/PBS (rabbit). Secondary antibody was biotinylated goat anti-hamster (CalTag) diluted 1:100 in 3% BSA/PBS/5% goat serum, or GARIG (Vector Labs, according to manufacturer's instructions). Staining was visualized using Vectastain Elite ABC (Vector Labs) and DAB/0.8% $NiCl_2$. Sections were counterstained with neutral red or methyl green and dehydrated. Immunoreactivity of both anti-α2 antibodies was confirmed by positive staining of mouse embryonic epidermis (data not shown).

RESULTS

Targeted Mutation of the Integrin α1 Gene by Homologous Recombination in Embryonic Stem Cells

The targeting vector containing 8 kb of α1 genomic sequence (Fig. 1) and a pgkneo polyA cassette inserted in reverse orientation at a unique *Cla*I site (see Materials and Methods) was electroporated into passage 8 J1 embryonic stem cells (Li *et al.*, 1992). Two hundred ES cell colonies were isolated after 10 days of selection with 125 μg/ml G418 and counterselection with 0.2 μM FIAU. Thirty clones showed homologous recombination by Southern analysis with a 200-bp external probe (Fig. 1), giving a homologous recombination efficiency of 15%. Five clones were injected into Balb/c E3.5 blastocysts. One clone gave rise to two chimeras which transmitted the targeted allele to the germline. Offspring were genotyped by PCR (see Materials and Methods). Intercrosses of heterozygotes gave rise to viable homozygous adults (Fig. 1) with a Mendelian distribution, in both mixed BALB/C 129sv and pure 129sv backgrounds (data not shown).

The Targeted Mutation Generated a Null Allele

In order to determine whether the α1 gene disruption generated a null allele, we analyzed mutant tissue and cells by immunohistochemistry and immunoprecipitation. Immunostaining of frozen sections of wild-type and homozygous mutant gut with a hamster anti-rat monoclonal anti-α1 antibody showed an absence of staining in homozygous mutant smooth muscle (Fig. 2). Embryonic fibroblasts (EFs) from E14.5 heterozygous and homozygous animals and smooth muscle cells (SMCs) from adult wild-type and homozygous animals were prepared. Immunoprecipitation of lysates of biotin surface labeled EFs was performed with the polyclonal anti-β1 antiserum K363 (Guan *et al.*, 1991). The 200-kDa α1 band was absent in homozygous EFs (Fig. 3) and SMCs. No aberrant bands were seen in immunoprecipitations of the mutant SMCs as compared to wild type, thus making it unlikely that a truncated functional α1 molecule formed a heterodimer with β1 (Fig. 3). Immunoprecipitation of EF lysates with a polyclonal antiserum against the cytoplasmic terminus of α1 failed to show any band in the mutants. Immunoprecipitations with antisera to α2 and α3 confirmed the presence of both molecules in EFs, with no obvious upregulation in the α1 deficient cells. Therefore neither a full-length nor a detectable truncated α1 molecule is present in the tissue of homozygotes for the α1 mutation.

Mice Lacking α1 Were Viable and Fecund

Homozygotes for the knockout allele showed no obvious alterations in behavior, size, or fecundity compared to wild-type and heterozygous littermates, in either mixed or 129sv backgrounds. Nor did they show any anatomic alterations at the gross level either during embryogenesis or adulthood (not shown). A histological survey showed no changes in the brain, heart, striated muscle, gut, lung, liver, kidney, or uterus of the mutants at the light microscopic level. Reticulin and Mallory's trichrome stains of liver showed no changes in the quantity or distribution of connective tissue in mutant liver (Fig. 4), suggesting that α1 is not required for the correct organization of matrix in this organ. Histological analysis of E6 embryos from a homozygous mating showed no abnormality in the distribution, polarity, or extent of invasion of trophoblast (Fig. 4), suggesting that α1 is not required for trophoblast invasion of the decidua. Homozygous mutant females had up to six litters of normal size and electron micrographs of mutant uterus showed no abnormality in the distribution of basement membranes between smooth muscle cells, or endothelial cells of small vessels, or in junctions of smooth muscle (Fig. 4). Thus our results failed to reveal any deficiency in the connective tissue or smooth muscle of α1 deficient mice.

Altered Adhesion of Mutant Cells in Vitro

In order to determine whether the adhesive properties of cells lacking integrin α1 were altered, embryonic fibroblasts and adult uterine smooth muscle cells were assayed for spreading on extracellular matrix proteins. Homozygous mutant EFs showed little spreading on collagen type IV 1 hr after plating, in contrast to the heterozygous EFs (Fig. 5). What little spreading that was observed was prevented by preincubation of the cells with an anti-α2 antibody. Consistent with this observation, spreading by heterozygous EFs was entirely inhibited by anti-α1 but not significantly by anti-α2. Thus, integrin α1 appears to be the primary receptor for collagen IV in EFs. The results for SMCs were somewhat different (see Fig. 6). Mutant and heterozygote SMCs showed similar spreading on collagen IV; however, preincubation with anti-α2 antibody entirely abrogated spreading on collagen IV in the mutant while leaving the wild-type cells essentially unaffected. Thus, in cultured smooth muscle cells, both α1 and α2

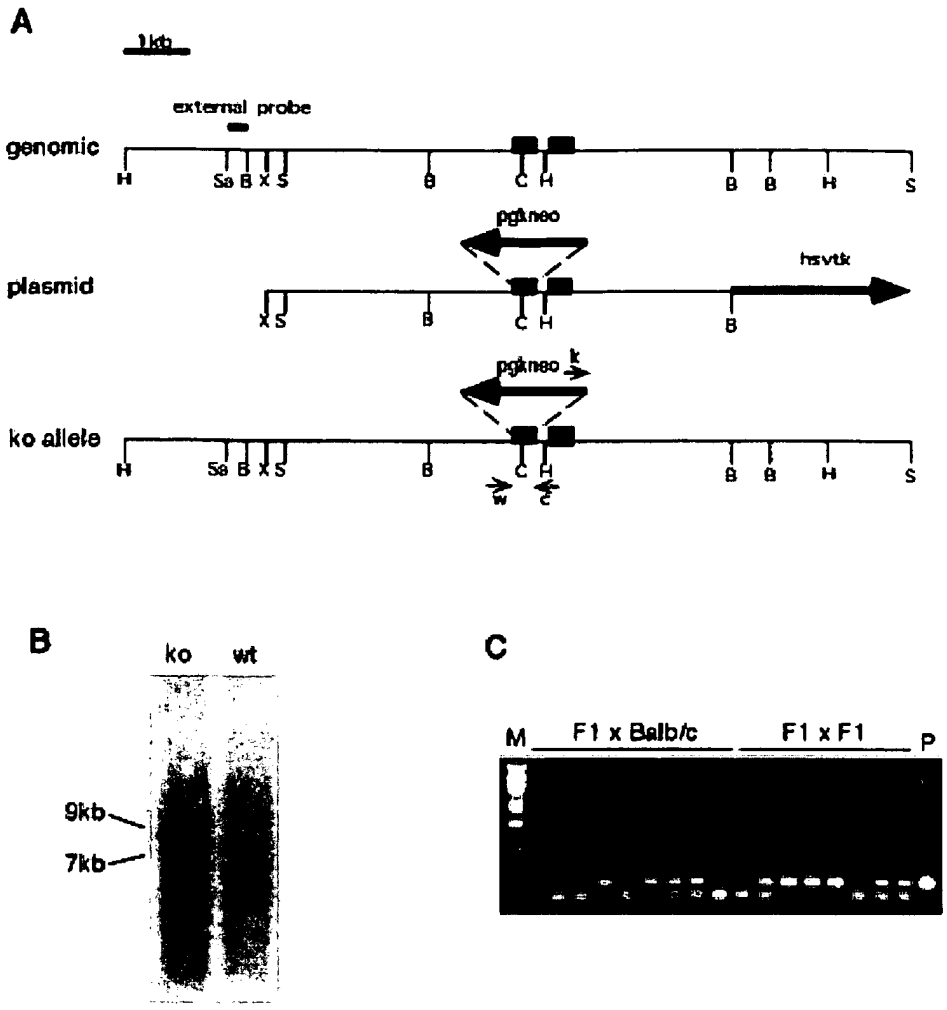


FIG. 1. (A) The α1 genomic clone, targeting vector pJKa1 ("plasmid"), and the expected mutant allele. Thick bars mark the two α1 exons, the 5′ one of which contains a unique *Cla*I site, into which is inserted a pgkneo polyA cassette, in reverse orientation, in the targeting construct. A TK cassette is present at the 3′ flank of the construct. The positions of the PCR genotyping primers w (wild type), k (mutant), and c (common) are indicated. Restriction enzymes: H, *Hin*dIII; Sa, *Sac*I; B, *Bam*HI; X, *Xho*I; S, *Sal*I; C, *Cla*I. (B) Representative Southern blot of wild type and targeted ES cell clones probed with the *Bam–Sac* fragment external probe after *Hin*dIII digestion. The wild-type band is approximately 7 kb and is lengthened to approximately 9 kb after insertion of the pgkneo cassette. (C) Representative PCR genotyping of tail DNA. The wild-type allele gives a 103-bp band, and the mutant a 273-bp band. Left bar indicates litter of heterozygous F1 male backcrossed to Balb/c; right bar shows the litter of an F1 heterozygote cross. P denotes PCR of the targeting construct. Note the presence of three homozygous mutant animals in the litter from the F1 × F1 cross.

appear to function as collagen IV receptors. Spreading on laminin at 10 μg/ml was similar in heterozygous and null EFs, but was reduced 50% by the addition of anti-α2 in null EFs. Heterozygous EFs were only slightly affected by anti-α2. Combination of anti-α1 and anti-α2 in heterozygous cells reduced spreading to that seen in null cells in the presence of anti-α2. Thus, α1 is a significant receptor for laminin in EFs. At a lower concentration of laminin (1.25 μg/ml), however, neither heterozygous nor mutant EFs showed significant spreading. This contrasted with the result seen in SMCs; spreading of wild-type and mutant SMCs was similar at 10 μg/ml laminin, but at 1.25 μg/ml mutant SMCs failed to spread while wild-type cells continued to do so. Therefore, α1 is a receptor for laminin in SMCs and appears to be more active as a laminin receptor when expressed by SMCs than when expressed by EFs. This result might reflect a genuine difference in α1 affinity, but might equally be due to the presence of other laminin receptors on SMCs which we have not examined. Spreading of EFs on collagen I was similar in heterozygous and mutant cells, even in the presence of anti-α2, which partially inhibited the spreading of both heterozygous and null EFs. This suggests that there is a third collagen I receptor in these cells and that α1 is not essential for

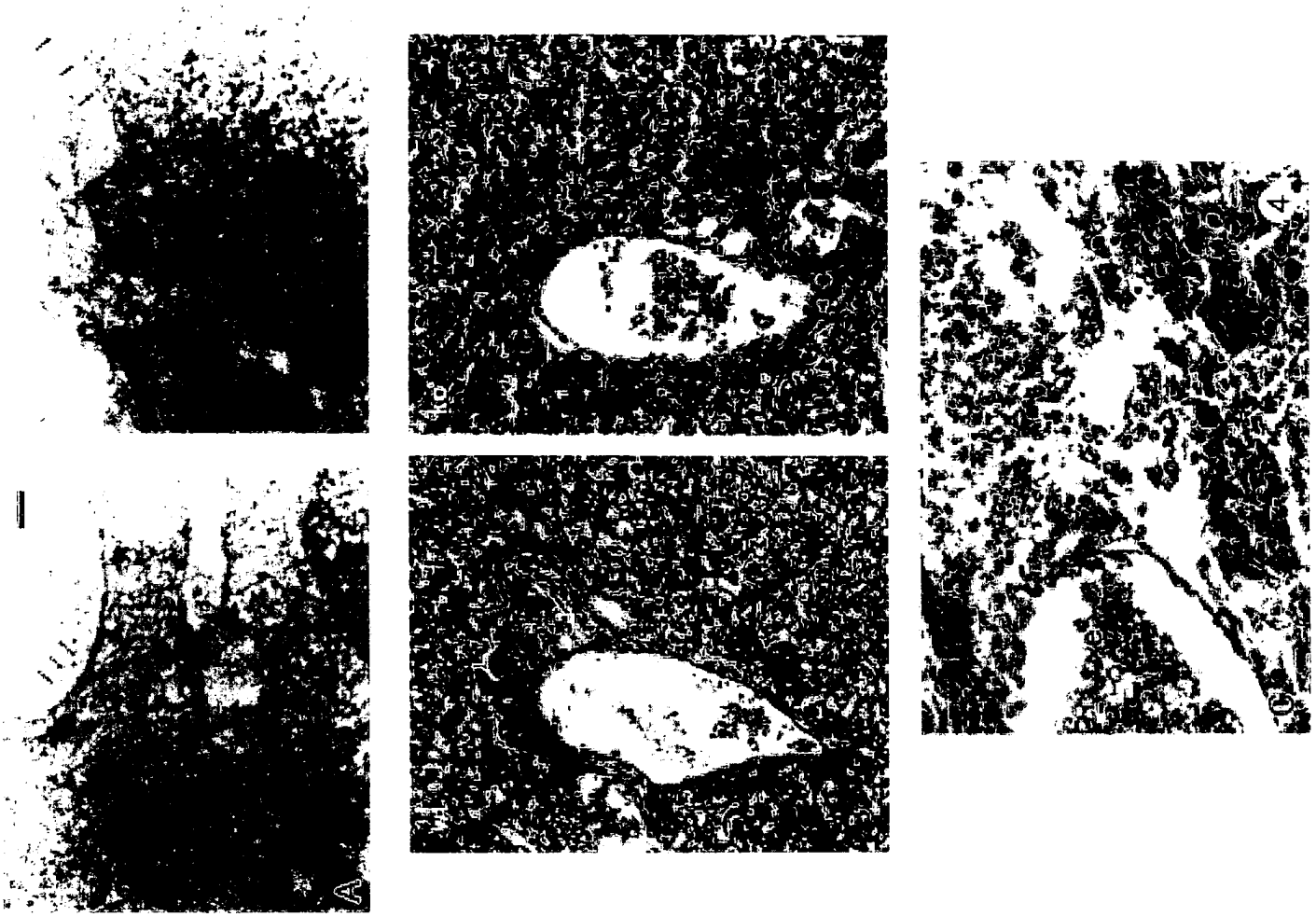


FIGURE 4

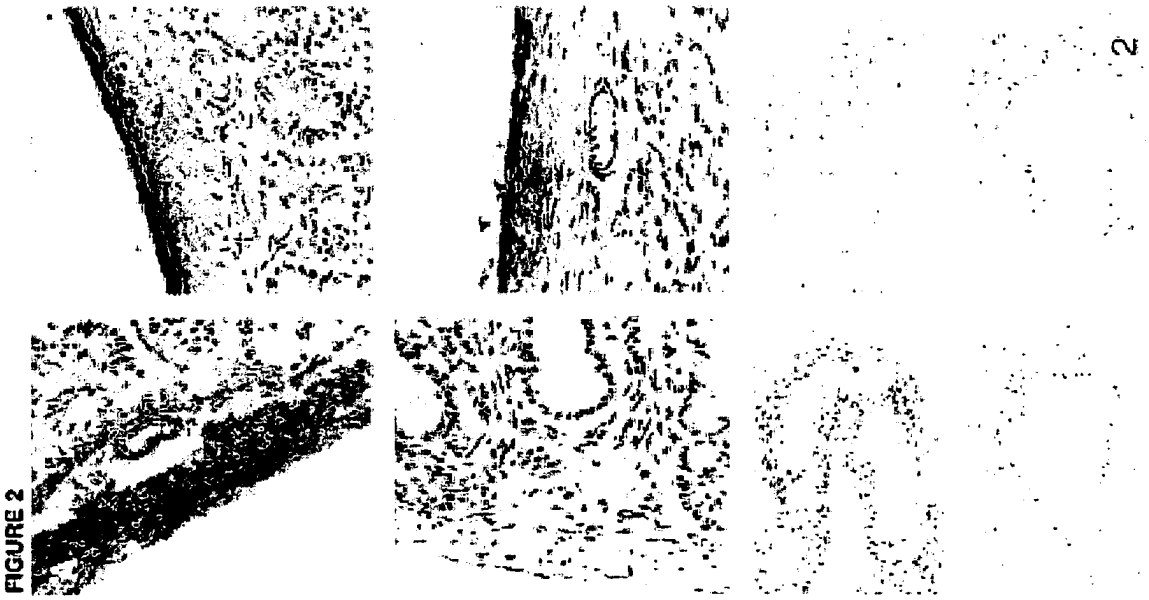


FIGURE 2

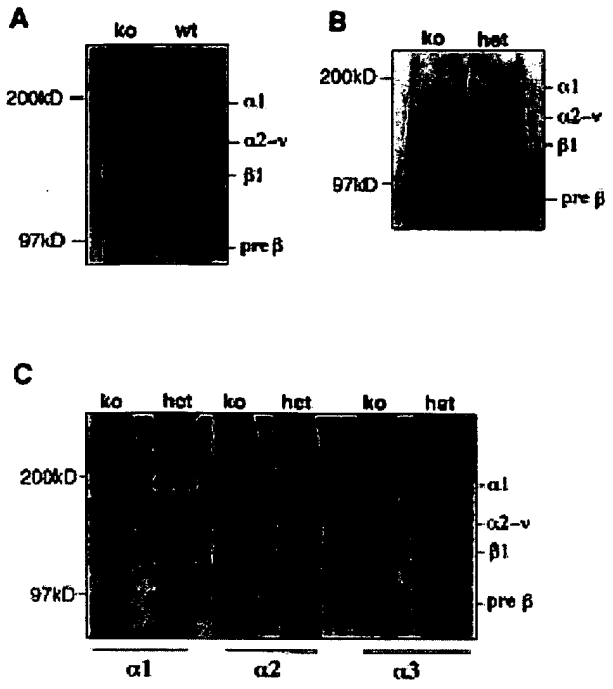


FIG. 3. Immunoprecipitation of surface biotinylated cells. The designation a2-v indicates the position of subunits α 2, 3, 4, 5, and v, which are not resolved from one another. (A) Extracts of smooth muscle cells from uteri of homozygote null (ko) and wild-type (wt) sibs, immunoprecipitated with the anti-β1 antibody K363. This antibody fails to coprecipitate an α1 band in the mutant. An aberrant band due to a truncated α 1 subunit is also not evident. (B) Extracts of embryonic fibroblasts from homozygous mutant (ko) and heterozygous (het) E13.5 sibs, immunoprecipitated with K363. Results are similar to those in A. (C) Extracts of embryonic fibroblasts as in B, immunoprecipitated with anti-α1 (left two lanes), anti-α2 (middle), and anti-α3 (right). α1 is absent in the mutant cells: α2 and α3 are present and similar in both. The anti-α3 does not generate a clear α3 band, but coprecipitates similar amounts of β1 in both cells of both genotypes.

collagen I binding. Thus, α1 is a receptor for collagen IV and laminin and is the primary receptor for collagen IV in EFs.

Altered Migration of Embryonic Fibroblasts into ECM Proteins

Although it is recognized that cell spreading and migration are related phenomena, it has also been suggested that the αv integrins are of primary importance in cell migration (Clyman *et al.*, 1992). We wished to test whether EFs are required $\alpha 1\beta 1$ for migration into collagen IV as well as for spreading on collagen IV, or whether another integrin could serve this purpose. We therefore employed a modified Boyden chamber haptotaxis assay to assay cell migration. α1 mutant primary and SV40 transformed EFs failed to migrate into collagen IV (Fig. 7), unlike their heterozygous counterparts, suggesting that α1 is also required for haptotaxis. Further analysis of migration of SV40 transformed EFs revealed that migration into laminin was slightly inhibited in the mutant cells, but migration into collagen I was unaffected. In both instances, addition of anti-α2 further reduced migration. Anti-α2 did not entirely prevent migration of mutant cells into collagen I. Anti-β1, however, completely inhibited migration. Thus, it appears that a third β1 integrin is functional in EFs for haptotaxis into, as well as spreading on, collagen I. Therefore, in EFs, $\alpha 1\beta 1$ is required for migration into collagen IV and is functional for migration into laminin, but is not required for migration into collagen I.

Compensation by $\alpha 2\beta 1$

Smooth muscle *in vivo* normally expresses $\alpha 1\beta 1$ but not $\alpha 2\beta 1$. Since the spreading assays demonstrated the presence of α2 in explanted passaged SMCs (consistent with the findings of Skinner *et al.* (1994)), we wished to determine whether the absence of α1 in the homozygote mutants was compensated for by expression of α2. Wild-type and homozygous gut were therefore analyzed for the presence of α2 by immunoperoxidase staining of frozen sections. Neither adult smooth muscle nor E16.5 esophagus showed a compensatory upregulation of α2 (Fig. 2). Therefore there is no evidence for lack of α1 causing induction of α2 *in vivo*.

FIG. 2. (A–D) Immunostain of wild-type (A and B) and homozygous mutant (C and D) gut for α1 (A, C) and α2 (polyclonal rabbit antiserum) (B, D). Objective, 10X. Note the absence of α1 immunostaining in the null animal, with abundant staining in the smooth muscle and vascular endothelium of the wild-type animal. The pattern of α2 staining, predominantly serosal, is similar in wild-type and α1 null animals. (E–H) Immunostain of heterozygous (E and F) and homozygous mutant (G and H) E14.5 esophagus for integrin α1 (E, G) and α2 (monoclonal hamster antiserum) (F, H). Abundant α1 is seen in the smooth muscle of the heterozygote, but is absent in the knockout. α2 immunostaining is similar in both animals, with light staining at the epithelial surface and none in the smooth muscle.

FIG. 4. (A) Electron micrographs of the junction between two uterine smooth muscle cells. (Scale bar, 100 nm) Left, wild type. Right, homozygous mutant. The distance between cells is similar, as is the distribution of dense areas suggesting junctions. Basal lamina (small arrows) is also similar in appearance. (B) Wild-type (wt) and homozygous mutant (ko) liver (40X objective, Mallory's trichrome stain). Hepatocytes, bile ducts, and overall architecture are similar in both animals. (C) E5 embryo and placenta from a homozygous mating (40× original magnification, H&E; e, embryo; c, cytotrophoblast; s, syncytiotrophoblast). Note normal trophoblast morphology with multinucleate syncytiotrophoblast invading the decidua.

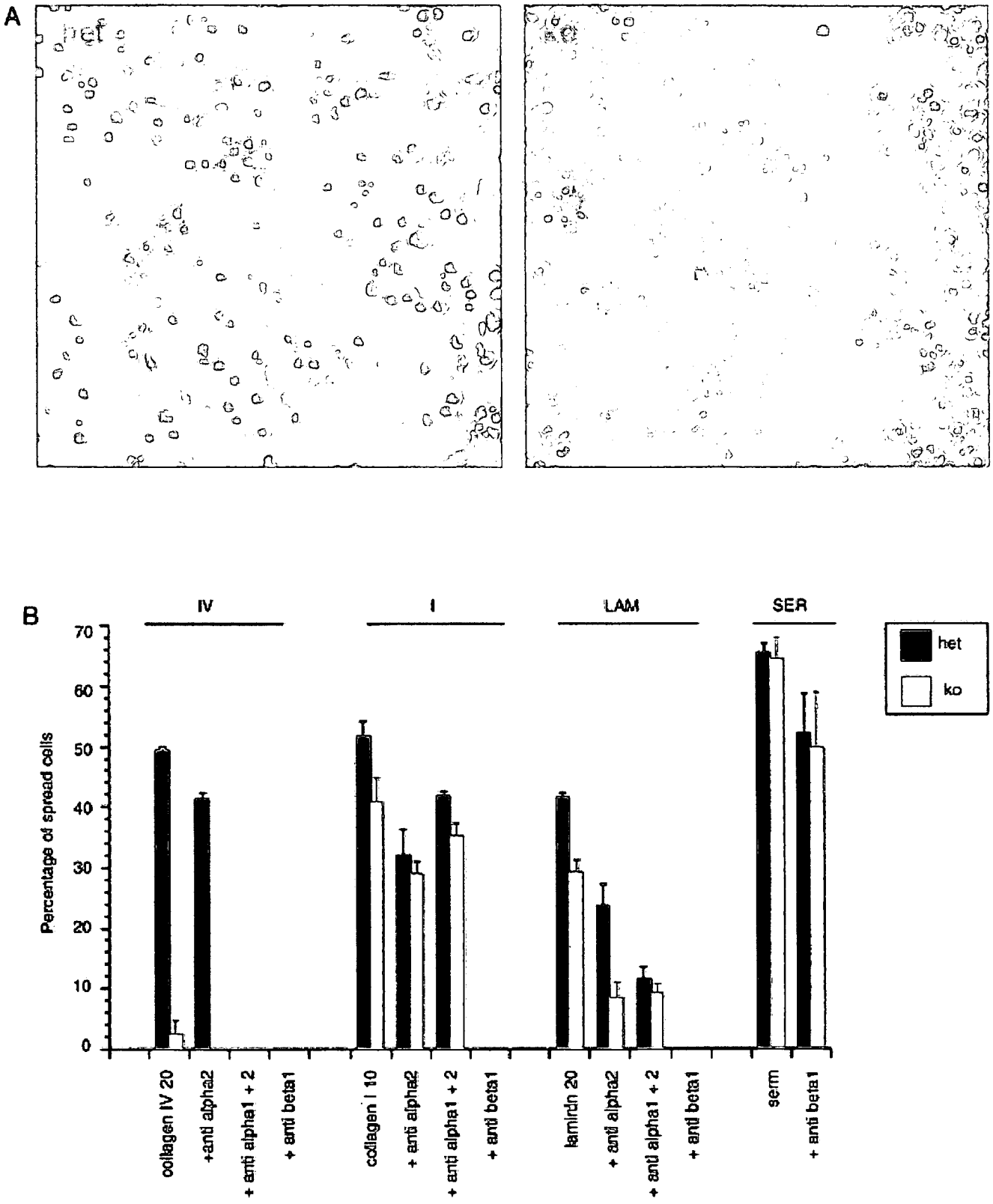


FIG. 5. (A) Spreading of heterozygous (left) and homozygous mutant (right) EFs on collagen IV (20 μg/ml). About half the cells are spread in the heterozygote; none are in the homozygote. (B) Representative spreading experiment on substrata of collagen IV (IV), collagen I (I), laminin (LAM), and serum (SER), with or without antibodies to $\alpha 2$, $\alpha 1 + \alpha 2$, or $\beta 1$. Substratum concentrations are indicated in μg/ml. Het indicates heterozygous EFs, and ko, homozygous mutant EFs.

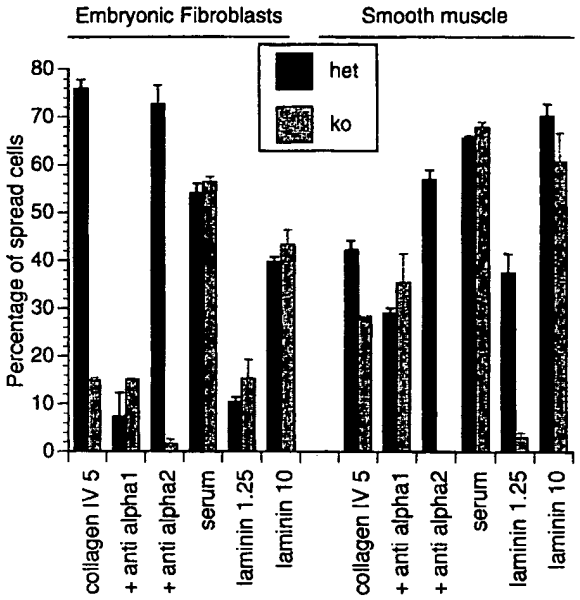


FIG. 6. Spreading of smooth muscle cells and embryonic fibroblasts on collagen IV with or without antibodies to $\alpha 1$ or $\alpha 2$, and on low and high concentrations of laminin. Substratum concentrations are indicated in μg/ml. Het indicates heterozygous EFs or wild-type SMCs, ko indicates homozygous mutant cells. In contrast to EFs, explanted smooth muscle cells can use $\alpha 2$ to spread on collagen IV and can use $\alpha 1$ to spread on very low concentrations of laminin.

DISCUSSION

We have generated mice with a null allele of the $\alpha 1$ integrin subunit by homologous recombination in embryonic stem cells. Mice homozygous for this allele were viable and fertile with no overt phenotype or anatomical abnormalities. The mutant allele generated a true null, as assayed by immunohistochemistry and immunoprecipitation. Smooth muscle of mutant animals did not show upregulation of the alternative collagen integrin receptor, $\alpha 2\beta 1$, either in the adult or in E16 embryos. Embryonic fibroblasts derived from mutant animals were unable to spread on collagen IV or migrate into this ligand in a haptotaxis assay, unlike those derived from heterozygotes. Deficiency of integrin $\alpha 1$ had a slight effect on spreading on laminin and little or no effect on spreading on collagen I. We therefore conclude that integrin $\alpha 1$ is primarily a receptor for collagen IV, and despite its prominent expression during development it is not essential for formation of the adult mouse.

Many observations had suggested the potential importance of $\alpha 1$ integrin in development, as is discussed in the Introduction. It was surprising, therefore, that no abnormalities in trophoblast invasion nor in later embryonic development have been observed in homozygous $\alpha 1$ null animals. It is possible that the molecule is redundant, or that its absence in development might be compensated for by overexpression of another integrin. Cells of the trophoblast use $\alpha 1$ for matrix invasion, but they also express $\alpha 6$, and a combination of antibodies to both $\alpha 1$ and $\alpha 6$ is required to block migration entirely (Damsky *et al.*, 1994). Therefore it is possible that interaction with laminin via $\alpha 6$ is all that is necessary for trophoblast invasion in the absence of interactions of $\alpha 1$ with matrix. $\alpha 6$ is also prominent in developing neurons, neural crest cells, skin, and the heart in development (Thorsteinsdottir *et al.*, 1995), and might provide any necessary adhesion to laminin. Skeletal muscle, which normally expresses $\alpha 1$ transiently, also expresses dystroglycan, a nonintegrin high-affinity laminin receptor, along with $\alpha 7$ (Mercurio, 1995), during development. Thus, as a laminin receptor, $\alpha 1$ may be redundant during embryogenesis. As a collagen receptor, however, $\alpha 1$ is unique in several sites, including smooth muscle and liver. As we have not found compensatory upregulation of $\alpha 2$, $\alpha 1$'s closest relative in structure and binding proclivities, in $\alpha 1$ null embryos, it is possible that either collagen–integrin interactions are not required in these tissues during embryogenesis, or that another integrin, possibly $\alpha 3$, has a greater role in collagen binding than previous work had suggested. Our observation that $\alpha 2$ is not upregulated in $\alpha 1$ null embryos is similar to observations of the fibronectin receptor null animals. The $\alpha 4\alpha 5$ double homozygous mutant embryo has an additive phenotype of the two single gene mutant animals (Joy Yang, personal communication), which suggests that one fibronectin receptor is not upregulated to compensate for the loss of the other. Therefore compensatory upregulation of one integrin to substitute for another has not been observed in integrin mutant animals analyzed so far.

Integrin $\alpha 1\beta 1$ has been implicated in the binding of various ligands, including collagen IV, laminin, and collagen I. Our results shed light upon the specificities of these interactions with $\alpha 1\beta 1$ as well as with other integrin heterodimers. The results with collagen I were the most complex: absence of $\alpha 1$ had little or no effect on EF spreading on collagen I, while addition of anti-$\alpha 2$ caused some reduction in cell spreading and anti-$\beta 1$ inhibited spreading completely. Therefore, while both $\alpha 1$ and $\alpha 2$ can bind to collagen I, their function on EFs as collagen I receptors appears to be redundant. Our results also implicate a third $\beta 1$ integrin as a receptor for collagen I in EFs. The most likely candidate is $\alpha 3\beta 1$, which is known to be present in cardiac fibroblasts and hepatocytes. In these cells it was not found to bind to columns of collagen I, and was therefore suggested not to be a receptor for collagen I (Gullberg *et al.*, 1992). Other studies, however, have implicated $\alpha 3\beta 1$ in collagen binding (Yamamoto and Yamamoto, 1994). We find that in EFs, $\alpha 1$ is only one of several receptors for laminin, but it appears to be the sole receptor for collagen IV. In SMCs, on the other hand, $\alpha 2$ is also a receptor for collagen IV. We also find that $\alpha 1$ appears to have a higher affinity for laminin in SMCs. These results suggest that the affinities of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ for their ligands may vary depending on the cell type in which they are expressed, an observation made also by

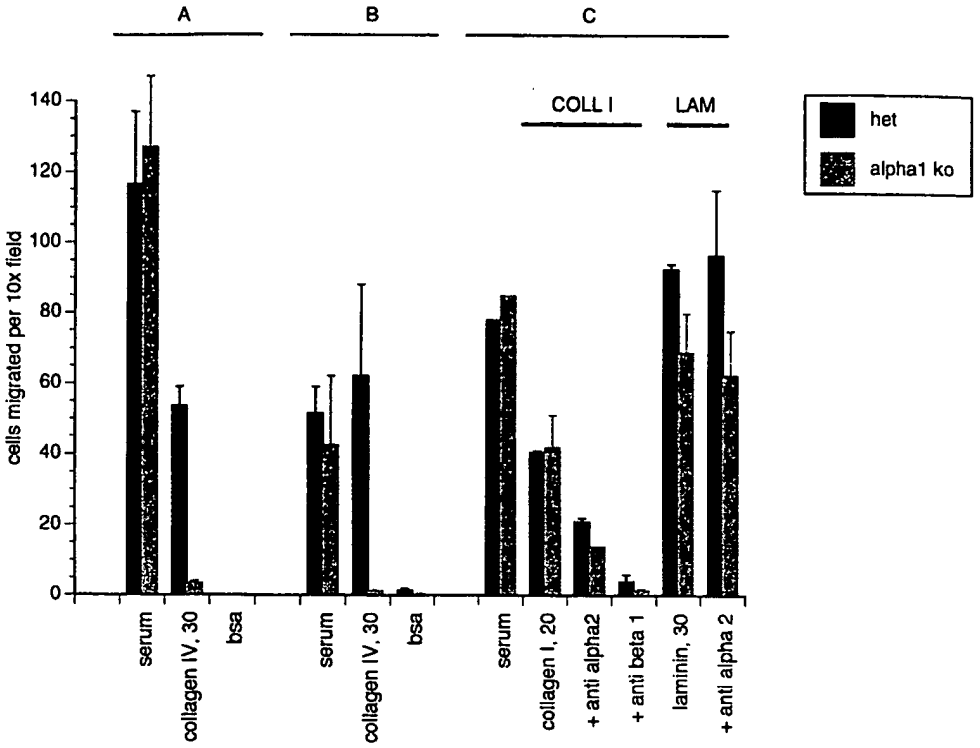


FIG. 7. Composite of three migration experiments. Substratum concentrations are indicated in μg/ml. Het designates heterozygous EFs, alpha1 ko, homozygous mutant EFs. (A) Migration of primary fibroblasts into collagen IV, serum, or BSA alone. Homozygous mutant EFs are unable to migrate into collagen IV. (B) Migration of SV40 transformed EFs, similar to A. Transformed homozygous mutant EFs are similarly unable to migrate into collagen IV. The reduced migration into serum relative to untransformed fibroblasts may be due to reduction in α5 synthesis (Plantefaber and Hynes, 1989). (C) Migration of transformed EFs into collagen I and laminin. Heterozygous and knockout EFs migrate similarly into collagen I and are similarly affected by anti α2. Anti-β1 blocks migration into collagen I. Migration into laminin is slightly inhibited in homozygous mutant cells, but is not affected by anti-α2.

Chan and Hemler (1993). Our observations of EF migration demonstrate that $\alpha 1\beta 1$ is necessary for haptotaxis into collagen IV; we have not determined whether the αv integrins, known for their promigratory properties, are also involved in this process.

To summarize the evidence, it appears that α1 is primarily a receptor for collagen IV rather than collagen I (Warren *et al.*, 1994), a hypothesis supported by our results. Also consistent with our findings in EFs is the observation of Tuckwell *et al.* (1995) that α2 has little affinity for collagen IV. These *in vitro* data are surprising given the distribution of the collagens and their receptors *in vivo*. Collagen IV is most abundant in the basement membranes of epithelia, while α1 is not found in epithelia; on the other hand, collagen I is abundant in smooth muscle, where α2 is absent. It appears, therefore, that integrin $\alpha 1\beta 1$ plays a more complex role than simply serving as an anchor for collagen.

Homozygote α1 mutant animals show no evidence of upregulation of integrin α2 in smooth muscle *in vivo*, while smooth muscle morphology in the mutant, including the presence of basal lamina, is unaltered. Furthermore, homozygous mutant females have normal litter sizes at normal frequency. Thus the absence of the major collagen binding integrin from smooth muscle *in vivo* does not appear to impair normal physiological function. As effective muscle contraction must require some force transmission through the extracellular matrix, our observations suggest that direct interaction between smooth muscle and collagen via integrins may not be required. It is striking that the $\alpha 8\beta 1$ integrin, a fibronectin receptor, exactly parallels $\alpha 1\beta 1$ in its distribution in adult tissues (Schnapp *et al.*, 1995). This observation, combined with our data, suggests that the interaction required for force transduction through collagen may in fact be mediated through fibronectin, which itself binds collagen (Ruoslahti *et al.*, 1982).

The presence of altered α1 expression in various disease states may provide some clues as to the function of the molecule in adulthood. Smooth muscle cells show upregulation of $\alpha 1\beta 1$ after balloon injury (Gotwals *et al.*, 1996), as they migrate to form neointima. CD8 T cells express $\alpha 1\beta 1$ while invading deep tissues, for example the synovium of arthritic joints. Thus $\alpha 1\beta 1$ may be important for cell migration in the repair of various forms of tissue injury. We are therefore examining the responses of α1 null mice to a vari-

ety of models of injury, including vascular injury, hepatic damage, wound healing, and hypertension. It has also been suggested that $\alpha 1\beta 1$ provides feedback inhibition of collagen synthesis, as a correlation between $\alpha 1$ deficiency and failure to downregulate collagen synthesis has been noted in the fibroblasts of patients with systemic sclerosis (Ivarsson *et al.*, 1993). We are examining this relationship in mutant EFs. The studies described here should help to define the roles of the $\alpha 1\beta 1$ receptor in the adult.

ACKNOWLEDGMENTS

We thank Guido Tarone, Sam Santoro, and Donna Mendrick for generous gifts of antibodies and Gene Marcantonio for the human $\alpha 1$ cDNA. H.G. thanks Jeanne Reis for histological slide preparation, Jessie Dausman for training in blastocyst injections, Ruth Curry for tail DNA preparation, Anne Vassalli and Kuo-Fen Lee for advice on culture and transfection of ES cells, and Brian Bates for reading the manuscript. This work was supported by NIH R35-CA44339 Outstanding Investigator and 5-POI-HL41484 Program of Excellence grants to R.J. H.G. was supported by an MRC (Canada) postdoctoral fellowship. V.E.K. is a Director of Research at INSERM.

REFERENCES

Belkin, V. M., Belkin, A. M. and Koteliansky, V. E. (1990). Human smooth muscle VLA-1 integrin: Purification, substrate specificity, localization in aorta, and expression during development. *J. Cell Biol.* 111, 2159-2170.

Briesewitz, R., Epstein, M. R., and Marcantonio, E. E. (1993). Expression of native and truncated forms of the human integrin alpha 1 subunit. *J. Biol Chem.* 268, 2989-2996.

Chan, B. M., and Hemler, M. E. (1993). Multiple functional forms of the integrin VLA-2 can be derived from a single alpha 2 cDNA clone: Interconversion of forms induced by an anti-beta 1 antibody. *J. Cell Biol.* 120, 537-543.

Clyman, R. I., Mauray, F., and Kramer, R. H. (1992). Beta 1 and beta 3 integrins have different roles in the adhesion and migration of vascular smooth muscle cells on extracellular matrix. *Exp. Cell Res.* 200, 272-284.

Colognato-Pyke, H., O'Rear, J. J., Yamada, Y., Carbonetto, S., Cheng, Y. S., and Yurchenco, P. D. (1995). Mapping of network-forming, heparin-binding, and alpha 1 beta 1 integrin-recognition sites within the alpha-chain short arm of laminin-1. *J. Biol. Chem.* 270, 9398-9406.

Damsky, C. H., Librach, C., Lim, K. H., Fitzgerald, M. L., McMaster, M. T., Janatpour, M., Zhou, Y., Logan, S. K., and Fisher, S. J. (1994). Integrin switching regulates normal trophoblast invasion. *Development* 120, 3657-3666.

Defilippi, P., van Hinsbergh, V., Bertolotto, A., Rossino, P., Silengo, L., and Tarone, G. (1991). Differential distribution and modulation of expression of alpha 1/beta 1 integrin on human endothelial cells. *J. Cell Biol.* 114, 855-863.

Duband, J. L., Belkin, A. M., Syfrig, J., Thiery, J. P., and Koteliansky, V. E. (1992). Expression of alpha 1 integrin, a laminin-collagen receptor, during myogenesis and neurogenesis in the avian embryo. *Development* 116, 585-600.

Erulkar, S. D., Rendt, J., Nori, R. D., and Ger, B. (1994). The influence of 17β-oestradiol on K^+ currents in smooth muscle cells isolated from immature rat uterus. *Proc. R. Soc. London B* 256, 59-65.

Fassler, R., and Meyer, M. (1995) Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev.* 9, 896-908.

Gardner, H. A., Berse, B., and Senger, D. R. (1994). Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts. *Oncogene* 9, 2321-2326.

George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* 119, 1079-1091.

Glukhova, M., Koteliansky, V., Fondacci, C., Marotte, F., and Rappaport, L. (1993). Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. *Dev. Biol.* 157, 437-447.

Glukhova, M., Koteliansky, V., Sastre, X., and Thiery, J. P. (1995). Adhesion systems in normal breast and in invasive breast carcinoma. *Am. J. Pathol.* 146, 706-716.

Gotwals, P. J., Chi-Rosso, G., Lindner, V., Yang, J., Ling, L., Fawell S. E., and Koteliansky, V. E. (1996). The $\alpha 1\beta 1$ integrin is expressed during neointima formation in rat arteries and mediates collagen matrix reorganisation. *J. Clin. Invest.*, in press.

Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991). Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul.* 2, 951-964.

Gui, G. P., Wells, C. A., Browne, P. D., Yeomans, P., Jordan, S., Puddefoot, J. R., Vinson, G. P., and Carpenter, R. (1995). Integrin expression in primary breast cancer and its relation to axillary nodal status. *Surgery* 117, 102-108.

Gullberg, D., Gehlsen, K. R., Turner, D. C., Ahlen, K., Zijenah, L. S., Barnes, M. J., and Rubin, K. (1992). Analysis of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 integrins in cell-collagen interactions: Identification of conformation dependent alpha 1 beta 1 binding sites in collagen type I. *EMBO J.* 11, 3865-3873.

Gullberg, D., Turner, D. C., Borg, T. K., Terracio, L., and Rubin, K. (1990). Different beta 1-integrin collagen receptors on rat hepatocytes and cardiac fibroblasts. *Exp. Cell Res.* 190, 254-264.

Hemler, M. E., Glass, D., Coblyn, J. S., and Jacobson, J. G. (1986). Very late activation antigens on rheumatoid synovial fluid T lymphocytes. Association with stages of T cell activation. *J. Clin. Invest.* 78, 696-702.

Hemler, M. E., Huang, C., and Schwarz, L. (1987). The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. *J. Biol. Chem.* 262, 3300-3309.

Hemler, M. E., and Jacobson, J. G. (1987). Cell matrix adhesion-related proteins VLA-1 and VLA-2: Regulation of expression on T cells. *J. Immunol.* 138, 2941-2948.

Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A., and Strominger, J. L. (1984). Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: Cell distribution and antigenic relation to components on resting cells and T cell lines. *J. Immunol.* 132, 3011-3018.

Hertle, M. D., Adams, J. C., and Watt, F. M. (1991). Integrin expression during human epidermal development in vivo and in vitro. *Development* 112, 193-206.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11-25.

Ignatius, M. J., Large, T. H., Houde, M., Tawil, J. W., Barton, A., Esch, F., Carbonetto, S., and Reichardt, L. F. (1990). Molecular cloning of the rat integrin alpha 1-subunit: A receptor for laminin and collagen. *J. Cell Biol.* 111, 709-720.

Ignatius, M. J., and Reichardt, L. F. (1988). Identification of a neuronal laminin receptor: An Mr 200K/120K integrin heterodimer that binds laminin in a divalent cation-dependent manner. *Neuron* 1, 713-725.

Ivarsson, M., McWhirter, A., Black, C. M., and Rubin, K. (1993). Impaired regulation of collagen pro-alpha 1(I) mRNA and change in pattern of collagen-binding integrins on scleroderma fibroblasts. *J. Invest. Dermatol.* 101, 216-221.

Janat, M. F., Argraves, W. S., and Liau, G. (1992). Regulation of vascular smooth muscle cell integrin expression by transforming growth factor beta1 and by platelet-derived growth factor-BB. *J. Cell Physiol.* 151, 588-595.

Juhasz, I., Murphy, G. F., Yan, H. C., Herlyn, M., and Albelda, S. M. (1993). Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous human wound healing in vivo. *Am. J. Pathol.* 143, 1458-1469.

Kern, A., Briesewitz, R., Bank, I., and Marcantonio, E. E. (1994). The role of the I domain in ligand binding of the human integrin alpha 1 beta 1. *J. Biol. Chem.* 269, 22811-22816.

Korhonen, M., Ylanne, J., Laitinen, L., and Virtanen, I. (1990a). The alpha 1-alpha 6 subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. *J. Cell Biol.* 111, 1245-1254.

Korhonen, M., Ylanne, J., Laitinen, L., and Virtanen, I. (1990b). Distribution of beta 1 and beta 3 integrins in human fetal and adult kidney. *Lab. Invest.* 62, 616-625.

Kramer, R. H., and Marks, N. (1989). Identification of integrin collagen receptors on human melanoma cells. *J. Biol. Chem.* 264, 4684-4688.

Kuhn, K., and Eble, J. (1994). The structural bases of integrin-ligand interactions. *Trends Cell Biol.* 4, 256-261.

Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* 19, 4293.

Lallier, T., and Bronner-Fraser, M. (1993). Inhibition of neural crest cell attachment by integrin antisense oligonucleotides. *Science* 259, 692-695.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, 915-926.

Liebert, M., Washington, R., Stein, J., Wedemeyer, G., and Grossman, H. B. (1994). Expression of the VLA beta 1 integrin family in bladder cancer. *Am. J. Pathol.* 144, 1016-1022.

Loeser, R. F., Carlson, C. S., and McGee, M. P. (1995). Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. *Exp. Cell Res.* 217, 248-257.

Mann, R., Mulligan, R. C., and Baltimore, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33, 153-159.

Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: A general strategy for targeting mutations to non-selectable genes. *Nature* 336, 348-352.

Mechtersheimer, G., Barth, T., Quentmeier, A., and Moller, P. (1994). Differential expression of beta 1 integrins in nonneoplastic smooth and striated muscle cells and in tumors derived from these cells. *Am. J. Pathol.* 144, 1172-1182.

Mercurio, A. (1995). Laminin receptors: Achieving specificity through cooperation. *Trends Cell Biol.* 5, 419-423.

Miyake, S., Sakurai, T., Okumura, K., and Yagita, H. (1994). Identification of collagen and laminin receptor integrins on murine T lymphocytes. *Eur. J. Immunol.* 24, 2000-2005.

Noakes, P. G., Gautam, M., Mudd, J., Sanes, J. R., and Merlie, J. P. (1995). Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. *Nature* 374, 258-262.

Olsen, B. (1995). New insights into the function of collagens from genetic analysis. *Curr. Opin. Cell Biol.* 7, 720-727.

Pasqualini, R., and Hemler, M. E. (1994). Contrasting roles for integrin beta 1 and beta 5 cytoplasmic domains in subcellular localization, cell proliferation, and cell migration. *J. Cell Biol.* 125, 447-460.

Perris, R., Syfrig, J., Paulsson, M., and Bronner-Fraser, M. (1993). Molecular mechanisms of neural crest cell attachment and migration on types I and IV collagen. *J. Cell Sci.* 106, 1357-1368.

Plantefaber, L. C., and Hynes, R. O. (1989). Changes in integrin receptors on oncogenically transformed cells. *Cell* 56, 281-290.

Rossino, P., Gavazzi, I., Timpl, R., Aumailley, M., Abbadini, M., Giancotti, F., Silengo, L., Marchisio, P. C., and Tarone, G. (1990). Nerve growth factor induces increased expression of a laminin-binding integrin in rat pheochromocytoma PC12 cells. *Exp. Cell Res.* 189, 100-108.

Roussel, E., Gingras, M. C., Ro, J. Y., Branch, C., and Roth, J. A. (1994). Loss of alpha 1 beta 1 and reduced expression of other beta 1 integrins and CAM in lung adenocarcinoma compared with pneumocytes. *J. Surg. Oncol.* 56, 198-208.

Rudnicki, M. A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* 71, 383-390.

Ruoslahti, E. (1991). Integrins. *J. Clin. Invest.* 87, 1-5.

Ruoslahti, E., Pierschbacher, M., Engvall, E., Oldberg, A., and Hayman, E. G. (1982). Molecular and biological interactions of fibronectin. *J. Invest. Dermatol.* 79, 65s-68s.

Schnapp, L. M., Breuss, J. M., Ramos, D. M., Sheppard, D., and Pytela, R. (1995). Sequence and tissue distribution of the human integrin alpha 8 subunit: A beta 1-associated alpha subunit expressed in smooth muscle cells. *J. Cell Sci.* 108, 537-544.

Schnieke, A., Harbers, K., and Jaenisch, R. (1983). Embryonic lethal mutation in mice induced by retrovirus insertion into the alpha 1(I) collagen gene. *Nature* 304, 315-320.

Serra, R., Carbonetto, S., Lord, M., and Isom, H. C. (1994). Transforming growth factor beta 1 suppresses transformation in hepatocytes by regulating alpha 1 beta 1 integrin expression. *Cell Growth Differ.* 5, 509-517.

Skinner, M. P., Raines, E. W., and Ross, R. (1994). Dynamic expression of alpha 1 beta 1 and alpha 2 beta 1 integrin receptors by human vascular smooth muscle cells. Alpha 2 beta 1 integrin is required for chemotaxis across type I collagen-coated membranes. *Am. J. Pathol.* 145, 1070-1081.

Sterry, W., Mielke, V., Konter, U., Kellner, I., and Boehncke, W. H. (1992). Role of beta 1-integrins in epidermotropism of malignant T cells. *Am. J. Pathol.* 141, 855-860.

Sutherland, A. E., Calarco, P. G., and Damsky, C. H. (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* 119, 1175-1186.

Suzuki, S., Takahashi, T., Nakamura, S., Koike, K., Ariyoshi, Y., Takahashi, T., and Ueda, R. (1993). Alterations of integrin expression in human lung cancer. *Jpn. J. Cancer Res.* 84, 168–174.

Tanaka, T., Ohtsuka, Y., Yagita, H., Shiratori, Y., Omata, M., and Okumura, K. (1995). Involvement of alpha1 and alpha4 integrins in gut mucosal injury of graft-versus-host disease. *Int. Immunol.* 7, 1183–1189.

Tawil, N. J., Houde, M., Blacher, R., Esch, F., Reichardt, L. F., Turner, D. C., and Carbonetto S. (1990). Alpha 1 beta 1 integrin heterodimer functions as a dual laminin/collagen receptor in neural cells. *Biochemistry* 29, 6540–6544.

Terracio, L., Rubin, K., Gullberg, D., Balog, E., Carver, W., Jyring, R., and Borg, T. K. (1991). Expression of collagen binding integrins during cardiac development and hypertrophy. *Circ. Res.* 68, 734–744.

Thorsteinsdottir, S., Roelen, B., Freund, E., Gaspar, A., Sonnenberg, A., and Mummery, C. (1995). Expression patterns of laminin receptor splice variants alpha6Abeta1 and alpha6Bbeta1 suggest different roles in development. *Dev. Dyn.* 204, 240–258.

Tomaselli, K. J., Doherty, P., Emmett, C. J., Damsky, C. H., Walsh, F. S., and Reichardt, L. F. (1993). Expression of beta 1 integrins in sensory neurons of the dorsal root ganglion and their functions in neurite outgrowth on two laminin isoforms. *J. Neurosci.* 13, 4880–4888.

Tuckwell, D., Calderwood, D. A., Green, L. J., and Humphries, M. J. (1995). Integrin alpha 2 I-domain is a binding site for collagens. *J. Cell Sci.* 108, 1629–1637.

Warren, A. P., Owens, C. N., Borysiewicz, L. K., and Patel, K. (1994). Down-regulation of integrin alpha 1/beta 1 expression and association with cell rounding in human cytomegalovirus-infected fibroblasts. *J. Gen. Virol.* 75, 3319–3325.

Wu, J. E., and Santoro, S. A. (1994). Complex patterns of expression suggest extensive roles for the alpha 2 beta 1 integrin in murine development. *Dev. Dyn.* 199, 292–314.

Xu, H., Wu, X. R., Wewer, U. M., and Engvall, E. (1994). Murine muscular dystrophy caused by a mutation in the laminin alpha 2 (Lama2) gene. *Nat. Genet.* 8, 297–302.

Yamamoto, K., and Yamamoto, M. (1994). Cell adhesion receptors for native and denatured type I collagens and fibronectin in rabbit arterial smooth muscle cells in culture. *Exp. Cell Res.* 214, 258–263.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1995). Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development* 121, 549–560.

Yang, J. T., Rayburn, H., and Hynes, R. O. (1993). Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* 119, 1093–1105.

Received for publication January 9, 1996
Accepted February 27, 1996

APPENDIX 6

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Listing of integrin-related publications of Humphrey Gardner (3 pages)

Listing of integrin-related publications of Thomas Krieg (3 pages)

NCBI

PubMed www.pubmed.gov

A service of the U.S. National Library of Medicine and the National Institutes of Health

My NCBI [Sign In] [Register]

All Databases | PubMed | Nucleotide | Protein | Genome | Structure | OMIM | PMC | Journals | Books

Search PubMed for gardner h and integrin Go Clear Save Search

Limits | Preview/Index | History | Clipboard | Details

Display Summary Show 20 Pub Date Send to

All: 20 Review: 1

Items 1 - 20 of 20 One page.

About Entrez
Text Version

Entrez PubMed
Overview
Help | FAQ
Tutorials
New/Noteworthy
E-Utilities

PubMed Services
Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
Special Queries
LinkOut
My NCBI

Related Resources
Order Documents
NLM Mobile
NLM Catalog
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

1: Louis H, Kakou A, Regnault V, Labat C, Bressenot A, Gao-Li J, Gardner H, Thornton SN, Challande P, Li Z, Lacolley P. Related Articles, Links

Role of alpha1beta1-integrin in arterial stiffness and angiotensin-induced arterial wall hypertrophy in mice.
Am J Physiol Heart Circ Physiol. 2007 Oct;293(4):H2597-604. Epub 2007 Jul 27.
PMID: 17660399 [PubMed - indexed for MEDLINE]

2: Conrad C, Boyman O, Tonel G, Tun-Kyi A, Laggner U, de Fougerolles A, Kotelianski V, Gardner H, Nestle FO. Related Articles, Links

Alpha1beta1 integrin is crucial for accumulation of epidermal T cells and the development of psoriasis.
Nat Med. 2007 Jul;13(7):836-42. Epub 2007 Jul 1.
PMID: 17603494 [PubMed - indexed for MEDLINE]

3: Chen L, Huq S, Gardner H, de Fougerolles AR, Barabino S, Dana MR. Related Articles, Links

Very late antigen 1 blockade markedly promotes survival of corneal allografts.
Arch Ophthalmol. 2007 Jun;125(6):783-8.
PMID: 17562989 [PubMed - indexed for MEDLINE]

4: Richter M, Ray SJ, Chapman TJ, Austin SJ, Rebhahn J, Mosmann TR, Gardner H, Kotelianski V, deFougerolles AR, Topham DJ. Related Articles, Links

Collagen distribution and expression of collagen-binding alpha1beta1 (VLA-1) and alpha2beta1 (VLA-2) integrins on CD4 and CD8 T cells during influenza infection.
J Immunol. 2007 Apr 1;178(7):4506-16.
PMID: 17372009 [PubMed - indexed for MEDLINE]

5: Hahm K, Lukashev ME, Luo Y, Yang WJ, Dolinski BM, Weinreb PH, Simon KJ, Chun Wang L, Leone DR, Lobb RR, McCrann DJ, Allaire NE, Horan GS, Fogo A, Kalluri R, Shield CF 3rd, Sheppard D, Gardner HA, Violette SM. Related Articles, Links

Alphav beta6 integrin regulates renal fibrosis and inflammation in Alport mouse.
Am J Pathol. 2007 Jan;170(1):110-25.
PMID: 17200187 [PubMed - indexed for MEDLINE]

6: Schapira K, Lutgens E, de Fougerolles A, Sprague A, Roemen A, Gardner H, Koteliansky V, Daemen M, Heeneman S. Related Articles, Links

Genetic deletion or antibody blockade of alpha1beta1 integrin induces a stable plaque phenotype in ApoE-/- mice.
Arterioscler Thromb Vasc Biol. 2005 Sep;25(9):1917-24. Epub 2005 Jun 23.
PMID: 15976328 [PubMed - indexed for MEDLINE]

7: Häkkinen L, Koivisto L, Gardner H, Saarialho-Kere U, Carroll JM, Lakso M, Rauvala H, Laato M, Heino J, Larjava H. Related Articles, Links

Increased expression of beta6-integrin in skin leads to spontaneous development of chronic wounds.
Am J Pathol. 2004 Jan;164(1):229-42.
PMID: 14695336 [PubMed - indexed for MEDLINE]

8: Ekholm E, Hankenson KD, Uusitalo H, Hiltunen A, Gardner H, Heino J, Penttinen R. Related Articles, Links

Diminished callus size and cartilage synthesis in alpha 1 beta 1 integrin-deficient mice during bone fracture healing.
Am J Pathol. 2002 May;160(5):1779-85.

PMID: 12000729 [PubMed - indexed for MEDLINE]

9: Szulgit G, Rudolph R, Wandel A, Tenenhaus M, Panos R, Gardner H. Related Articles, Links

Alterations in fibroblast alpha1beta1 integrin collagen receptor expression in keloids and hypertrophic scars.

J Invest Dermatol. 2002 Mar;118(3):409-15.
PMID: 11874478 [PubMed - indexed for MEDLINE]

10: Pozzi A, LeVine WF, Gardner HA. Related Articles, Links

Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis.

Oncogene. 2002 Jan 10;21(2):272-81.
PMID: 11803470 [PubMed - indexed for MEDLINE]

11: Cosgrove D, Rodgers K, Meehan D, Miller C, Bovard K, Gilroy A, Gardner H, Kotelianski V, Gotwals P, Amatucci A, Kalluri R. Related Articles, Links

Integrin alpha1beta1 and transforming growth factor-beta1 play distinct roles in alport glomerular pathogenesis and serve as dual targets for metabolic therapy.

Am J Pathol. 2000 Nov;157(5):1649-59.
PMID: 11073824 [PubMed - indexed for MEDLINE]

12: Wagner S, Gardner H. Related Articles, Links

Modes of regulation of laminin-5 production by rat astrocytes.

Neurosci Lett. 2000 Apr 21;284(1-2):105-8.
PMID: 10771173 [PubMed - indexed for MEDLINE]

13: Meharra EJ, Schön M, Hassett D, Parker C, Havran W, Gardner H. Related Articles, Links

Reduced gut intraepithelial lymphocytes in VLA1 null mice.

Cell Immunol. 2000 Apr 10;201(1):1-5.
PMID: 10805967 [PubMed - indexed for MEDLINE]

14: de Fougerolles AR, Sprague AG, Nickerson-Nutter CL, Chi-Rosso G, Rennert PD, Gardner H, Gotwals PJ, Lobb RR, Koteliansky VE. Related Articles, Links

Regulation of inflammation by collagen-binding integrins alpha1beta1 and alpha2beta1 in models of hypersensitivity and arthritis.

J Clin Invest. 2000 Mar;105(6):721-9.
PMID: 10727440 [PubMed - indexed for MEDLINE]

15: Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA. Related Articles, Links

Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization.

Proc Natl Acad Sci U S A. 2000 Feb 29;97(5):2202-7.
PMID: 10681423 [PubMed - indexed for MEDLINE]

16: Gardner HA. Related Articles, Links

Integrin signaling in fibrosis and scleroderma.

Curr Rheumatol Rep. 1999 Oct;1(1):28-33. Review.
PMID: 11123011 [PubMed - indexed for MEDLINE]

17: Gardner H, Broberg A, Pozzi A, Laato M, Heino J. Related Articles, Links

Absence of integrin alpha1beta1 in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis.

J Cell Sci. 1999 Feb;112 (Pt 3):263-72.
PMID: 9885280 [PubMed - indexed for MEDLINE]

18: Pozzi A, Wary KK, Giancotti FG, Gardner HA. Related Articles, Links

Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo.

J Cell Biol. 1998 Jul 27;142(2):587-94.
PMID: 9679154 [PubMed - indexed for MEDLINE]

19: Gardner H, Kreidberg J, Koteliansky V, Jaenisch R. Related Articles, Links

Deletion of integrin alpha 1 by homologous recombination permits normal murine development but gives rise to a specific deficit in cell adhesion.

Dev Biol. 1996 May 1;175(2):301-13.

20: Gardner HA, Berse B, Senger DR.

Related Articles, Links

Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts.

Oncogene. 1994 Aug;9(8):2321-6.
PMID: 8036014 [PubMed - indexed for MEDLINE]

Items 1 - 20 of 20

One page.

Display Summary Show 20 Pub Date Send to

NCBI

PubMed www.pubmed.gov

A service of the U.S. National Library of Medicine and the National Institutes of Health

My NCBI [Sign In] [Register]

All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Books

Search PubMed for krieg t and integrin Go Clear Save Search

Limits Preview/Index History Clipboard Details

About Entrez
Text Version

Entrez PubMed
Overview
Help | FAQ
Tutorials
New/Noteworthy
E-Utilities

PubMed Services
Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
Special Queries
LinkOut
My NCBI

Related Resources
Order Documents
NLM Mobile
NLM Catalog
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

Display Summary Show 50 Pub Date Send to

All: 29 Review: 4

Items 1 - 29 of 29 One page.

1: Woodall BP, Nystrom A, Iozzo RA, Eble JA, Niland S, Krieg T, Eckes B, Pozzi A, Iozzo RV. Related Articles, Links

Integrin alpha 2beta 1 is the required receptor for endorepellin angiostatic activity.
J Biol Chem. 2007 Nov 15; [Epub ahead of print]
PMID: 18024432 [PubMed - as supplied by publisher]

2: Zweers MC, Davidson JM, Pozzi A, Hallinger R, Janz K, Quondamatteo F, Leutgeb B, Krieg T, Eckes B. Related Articles, Links

Integrin alpha2beta1 is required for regulation of murine wound angiogenesis but is dispensable for reepithelialization.
J Invest Dermatol. 2007 Feb;127(2):467-78. Epub 2006 Sep 14.
PMID: 16977325 [PubMed - indexed for MEDLINE]

3: Kess D, Lindqvist AK, Peters T, Wang H, Zamek J, Nischt R, Broman KW, Blakytny R, Krieg T, Holmdahl R, Scharffetter-Kochanek K. Related Articles, Links

Identification of susceptibility loci for skin disease in a murine psoriasis model.
J Immunol. 2006 Oct 1;177(7):4612-9.
PMID: 16982899 [PubMed - indexed for MEDLINE]

4: Zweers MC, Siewe L, Wickenhauser C, Krieg T, Roers A, Eckes B. Related Articles, Links

Integrin alpha2beta1 deficiency does not affect contact hypersensitivity.
Arch Dermatol Res. 2006 Sep;298(4):201-5. Epub 2006 Aug 5.
PMID: 16897075 [PubMed - indexed for MEDLINE]

5: Eckes B, Zweers MC, Zhang ZG, Hallinger R, Mauch C, Aumailley M, Krieg T. Related Articles, Links

Mechanical tension and integrin alpha 2 beta 1 regulate fibroblast functions.
J Investig Dermatol Symp Proc. 2006 Sep;11(1):66-72. Review.
PMID: 17069012 [PubMed - indexed for MEDLINE]

6: Peters T, Bloch W, Wickenhauser C, Tawadros S, Oreshkova T, Kess D, Krieg T, Müller W, Scharffetter-Kochanek K. Related Articles, Links

Terminal B cell differentiation is skewed by deregulated interleukin-6 secretion in beta2 integrin-deficient mice.
J Leukoc Biol. 2006 Sep;80(3):599-607. Epub 2006 Jul 14.
PMID: 16844762 [PubMed - indexed for MEDLINE]

7: Klose A, Wilbrand-Hennes A, Zigrino P, Weber E, Krieg T, Mauch C, Hunzelmann N. Related Articles, Links

Contact of high-invasive, but not low-invasive, melanoma cells to native collagen I induces the release of mature cathepsin B.
Int J Cancer. 2006 Jun 1;118(11):2735-43.
PMID: 16381007 [PubMed - indexed for MEDLINE]

8: Zinn M, Aumailley M, Krieg T, Smola H. Related Articles, Links

Expression of laminin 5 by parental and c-Ha-ras-transformed HaCaT keratinocytes in organotypic cultures.
Eur J Cell Biol. 2006 May;85(5):333-43. Epub 2006 Feb 7.
PMID: 16460839 [PubMed - indexed for MEDLINE]

9: Zhang ZG, Bothe I, Hirche F, Zweers M, Gullberg D, Pfitzer G, Krieg T, Eckes B, Aumailley M. Related Articles, Links

Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of

alpha2beta1 integrin.
J Cell Sci. 2006 May 1;119(Pt 9):1886-95.
PMID: 16636073 [PubMed - indexed for MEDLINE]

10: Peters T, Sindrilaru A, Hinz B, Hinrichs R, Menke A, Al-Azzeh EA, Holzwarth K, Oreshkova T, Wang H, Kess D, Walzog B, Sulyok S, Sunderkötter C, Friedrich W, Wlaschek M, Krieg T, Scharffetter-Kochanek K. Related Articles, Links

Wound-healing defect of CD18(-/-) mice due to a decrease in TGF-beta1 and myofibroblast differentiation.
EMBO J. 2005 Oct 5;24(19):3400-10. Epub 2005 Sep 8.
PMID: 16148944 [PubMed - indexed for MEDLINE]

11: Grüner S, Prostredna M, Aktas B, Moers A, Schulte V, Krieg T, Offermanns S, Eckes B, Nieswandt B. Related Articles, Links

Anti-glycoprotein VI treatment severely compromises hemostasis in mice with reduced alpha2beta1 levels or concomitant aspirin therapy.
Circulation. 2004 Nov 2;110(18):2946-51. Epub 2004 Oct 25.
PMID: 15505105 [PubMed - indexed for MEDLINE]

12: Grüner S, Prostredna M, Schulte V, Krieg T, Eckes B, Brakebusch C, Nieswandt B. Related Articles, Links

Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo.
Blood. 2003 Dec 1;102(12):4021-7. Epub 2003 Jul 31.
PMID: 12893753 [PubMed - indexed for MEDLINE]

13: Kess D, Peters T, Zamek J, Wickenhauser C, Tawadros S, Loser K, Varga G, Grabbe S, Nischt R, Sunderkötter C, Müller W, Krieg T, Scharffetter-Kochanek K. Related Articles, Links

CD4+ T cell-associated pathophysiology critically depends on CD18 gene dose effects in a murine model of psoriasis.
J Immunol. 2003 Dec 1;171(11):5697-706.
PMID: 14634077 [PubMed - indexed for MEDLINE]

14: Noszczyk BH, Klein E, Holtkoetter O, Krieg T, Majewski S. Related Articles, Links

Integrin expression in the dermis during scar formation in humans.
Exp Dermatol. 2002 Aug;11(4):311-8.
PMID: 12190939 [PubMed - indexed for MEDLINE]

15: Holtkötter O, Nieswandt B, Smyth N, Müller W, Hafner M, Schulte V, Krieg T, Eckes B. Related Articles, Links

Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen.
J Biol Chem. 2002 Mar 29;277(13):10789-94. Epub 2002 Jan 11.
PMID: 11788609 [PubMed - indexed for MEDLINE]

16: Tunggal L, Ravaux J, Pesch M, Smola H, Krieg T, Gaill F, Sasaki T, Timpl R, Mauch C, Aumailley M. Related Articles, Links

Defective laminin 5 processing in cylindroma cells.
Am J Pathol. 2002 Feb;160(2):459-68.
PMID: 11839566 [PubMed - indexed for MEDLINE]

17: Niland S, Cremer A, Fluck J, Eble JA, Krieg T, Sollberg S. Related Articles, Links

Contraction-dependent apoptosis of normal dermal fibroblasts.
J Invest Dermatol. 2001 May;116(5):686-92.
PMID: 11348456 [PubMed - indexed for MEDLINE]

18: Herzhoff K, Sollberg S, Huerkamp C, Krieg T, Eckes B. Related Articles, Links

Fibroblast expression of collagen integrin receptors alpha1beta1 and alpha2beta1 is not changed in systemic scleroderma.
Br J Dermatol. 1999 Aug;141(2):218-23.
PMID: 10468791 [PubMed - indexed for MEDLINE]

19: Eckes B, Kessler D, Aumailley M, Krieg T. Related Articles, Links

Interactions of fibroblasts with the extracellular matrix: implications for the understanding of fibrosis.
Springer Semin Immunopathol. 1999;21(4):415-29. Review.

PMID: 10945034 [PubMed - indexed for MEDLINE]

20: Scharffetter-Kochanek K, Klein P, Krieg T. Related Articles, Links

Cellular and molecular mechanisms of tissue repair.

Basic Res Cardiol. 1998;93 Suppl 3:1-3. Review. No abstract available.
PMID: 9879434 [PubMed - indexed for MEDLINE]

21: Langholz O, Roeckel D, Petersohn D, Broermann E, Eckes B, Krieg T. Related Articles, Links

Cell-matrix interactions induce tyrosine phosphorylation of MAP kinases ERK1 and ERK2 and PLCgamma-1 in two-dimensional and three-dimensional cultures of human fibroblasts.

Exp Cell Res. 1997 Aug 25;235(1):22-7.
PMID: 9281348 [PubMed - indexed for MEDLINE]

22: Kozlowska E, Sollberg S, Mauch C, Eckes B, Klein CE, Krieg T. Related Articles, Links

Decreased expression of alpha 2 beta 1 integrin in scleroderma fibroblasts.

Exp Dermatol. 1996 Feb;5(1):57-63.
PMID: 8624613 [PubMed - indexed for MEDLINE]

23: Korge BP, Krieg T. Related Articles, Links

The molecular basis for inherited bullous diseases.

J Mol Med. 1996 Feb;74(2):59-70. Review.
PMID: 8820401 [PubMed - indexed for MEDLINE]

24: Langholz O, Röckel D, Mauch C, Kozlowska E, Bank I, Krieg T, Eckes B. Related Articles, Links

Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins.

J Cell Biol. 1995 Dec;131(6 Pt 2):1903-15.
PMID: 8557756 [PubMed - indexed for MEDLINE]

25: Eckes B, Krieg T, Nusgens BV, Lapière CM. Related Articles, Links

In vitro reconstituted skin as a tool for biology, pharmacology and therapy: a review.

Wound Repair Regen. 1995 Jul-Sep;3(3):248-57.
PMID: 17173550 [PubMed]

26: Lange TS, Bielinsky AK, Kirchberg K, Bank I, Herrmann K, Krieg T, Scharffetter-Kochanek K. Related Articles, Links

Mg2+ and Ca2+ differentially regulate beta 1 integrin-mediated adhesion of dermal fibroblasts and keratinocytes to various extracellular matrix proteins.

Exp Cell Res. 1994 Sep;214(1):381-8.
PMID: 7521847 [PubMed - indexed for MEDLINE]

27: Scharffetter-Kochanek K, Klein CE, Heinen G, Mauch C, Schaefer T, Adelmann-Grill BC, Goerz G, Fusenig NE, Krieg TM, Plewig G. Related Articles, Links

Migration of a human keratinocyte cell line (HACAT) to interstitial collagen type I is mediated by the alpha 2 beta 1-integrin receptor.

J Invest Dermatol. 1992 Jan;98(1):3-11.
PMID: 1728638 [PubMed - indexed for MEDLINE]

28: Klein CE, Dressel D, Steinmayer T, Mauch C, Eckes B, Krieg T, Bankert RB, Weber L. Related Articles, Links

Integrin alpha 2 beta 1 is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils.

J Cell Biol. 1991 Dec;115(5):1427-36.
PMID: 1955483 [PubMed - indexed for MEDLINE]

29: Majewski S, Hunzelmann N, Johnson JP, Jung C, Mauch C, Ziegler-Heitbrock HW, Riethmüller G, Krieg T. Related Articles, Links

Expression of intercellular adhesion molecule-1 (ICAM-1) in the skin of patients with systemic scleroderma.

J Invest Dermatol. 1991 Oct;97(4):667-71.
PMID: 1682392 [PubMed - indexed for MEDLINE]

Items 1 - 29 of 29 One page.

Display Summary Show 50 Pub Date Send to

APPENDIX 7

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Van der Vieren et al., Immunity. 1995 Dec;3(6):683-90. (see Appendix 4)

Kumar, Oncogene. 1998 Sep 17;17:1365-73. (9 pages)

Oncogene (1998) 17, 1365-1373

http://www.stockton-press.co.uk/onc

Signaling by integrin receptors

C Chandra Kumar

Department of Tumor Biology, Schering-Plough Research Institute, 2015, Galloping Hill Road, Kenilworth, New Jersey 07033, USA

Adhesive interactions are critical for the proliferation, survival and function of all cells. Integrin receptors as the major family of adhesion receptors have been the focus of study for more than a decade. These studies have tremendously enhanced our understanding of the integrin-mediated adhesive interactions and have unraveled novel integrin functions in cell survival mechanisms and in the activation of divergent signaling pathways. The signals from integrin receptors are integrated from those originating from growth factor receptors in order to organize the cytoskeleton, stimulate cell proliferation and rescue cells from matrix detachment-induced programmed cell death. These functions are critical in the regulation of multiple processes such as tissue development, inflammation, angiogenesis, tumor cell growth and metastasis and programmed cell death.

Keywords: integrins; cell adhesion; cell survival; focal adhesion complexes

Introduction

The term Integrin was coined about a decade ago to describe cell surface receptors that mediate cellular adhesion to extracellular matrix proteins and to other cells (Tamkun *et al.*, 1986; Ruoslahti and Pierschbacher, 1987). However, it has become clear that the biological significance of integrin-mediated adhesive events goes beyond simple physical linkage of cells to extracellular matrix proteins or to other cells. Adhesion is shown to be important for survival and proliferation of various cell types (Hynes, 1994; Ruoslahti and Reed, 1994; Meredith *et al.*, 1993; Meredith and Schwartz, 1997). A remarkable number of classical signaling pathways are now known to be activated by interactions of cells with matrix proteins via integrins (Juliano and Haskill, 1993; Schwartz *et al.*, 1995; Clarke and Brugge, 1995). Regulation of adhesion by integrins is also central to a number of specialized pathways in the hematopoietic systems, allowing attachment of platelets to soluble ligands and of lymphocytes to antigen-presenting cells and the phagocytosis of complement opsonized targets by granulocytes and macrophages. Cell-matrix interactions result in a cascade of cellular responses which ultimately promote not only cell binding but also lead to cell spreading. Both cell attachment and spreading are critical for cell survival because cells that adhere, but can not spread, may undergo programmed cell death (Bates *et al.*, 1994; Re *et al.*, 1994)

Integrin receptors are composed of noncovalently associated α and β chains which form heterodimeric receptor complexes (Hynes, 1992; Ruoslahti, 1991, 1996). The α and β subunits contain a large extracellular domain, a short transmembrane domain and a cytoplasmic carboxy terminal domain of variable length. The extracellular domains of both the α and β chains form the ligand binding domain. In mammals, 17 α subunits and 8 β subunits are known. These α and β subunits heterodimerize to produce 22 different receptors (Figure 1). Despite this high degree of redundancy, most integrins seem to have specific biological functions raising the possibility of signaling differences between integrins. Integrins not only bind ligands present in the extracellular matrix such as fibronectin, collagen, vitronectin etc., certain integrins can also bind to soluble ligands such as fibrinogen or to counter-receptors such as intracellular adhesion molecules (ICAMs) on adjacent cells.

Many of the integrins recognize the RGD (Arg-Gly-Asp) sequence in their matrix ligands (Ruoslahti, 1996). Nevertheless, they are capable of distinguishing different RGD-containing proteins such that some bind primarily to fibronectin and others to vitronectin. Integrins are also expressed in a cell-type specific manner; thus one group of integrins such as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$ is associated with migration and proliferation in various cell types. Many other integrins are expressed in selective cell types. Examples of cell-type specific integrins include $\alpha_{IIb}\beta_3$ in platelets and $\alpha_6\beta_4$ in epithelial cells. Integrins are capable of generating both common signals and signals specific for individual integrins. The signals from these integrin receptors are integrated with those originating from growth factor and cytokine receptors in order to organize the cytoskeleton, stimulate mitogen activated protein (MAP) kinase cascades and regulate immediately early gene expression. It has also been recognized that information can also flow in the reverse direction; the integrin ligand binding activity is also regulated from the inside of the cell (Schwartz *et al.*, 1995; Kolamus and Seed, 1997). Thus integrin receptors are highly versatile proteins mediating a variety of biological processes.

In this review, I will summarize the recent studies on three signaling pathways activated by integrin receptors. Specifically the role of integrins in (1) cytoskeletal organization, (2) in cell proliferation signaling pathways and (3) in cell survival pathways will be reviewed. A brief summary of the role of integrins in cell transformation and therapeutic applications is also included.

Role of integrins in cytoskeletal organization

The binding of ligands to integrin receptors leads to cross-linking or clustering of integrins. This promotes

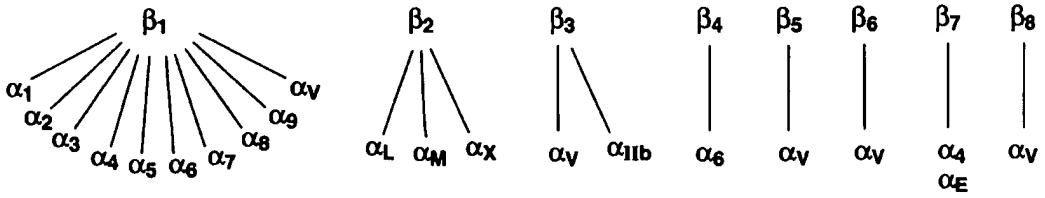


Figure 1 $\alpha\beta$ pairings of integrin receptors

the formation of structures at the cell membrane known as focal adhesions where integrins link the outside matrix to intracellular cytoskeletal complexes (Burridge *et al.*, 1988). These protein assemblies play an important role in modulating cell adhesion and inducing cell shape changes involved in cell spreading and locomotion. Focal adhesion complexes are readily seen by immunofluoresence as tear shaped plaques at the ends of actin filament based stress fibers. A diverse number of structural and signaling proteins, such as integrins, cytoskeletal proteins, protein kinases and signaling molecules are known to be concentrated at these sites (Craig and Johnson, 1996; Yamada and Miyamoto, 1995; Lo and Chen, 1994) (Figure 2). Actin binding proteins that colocalize with integrins at focal adhesion plaques include, α-actinin, talin, tensin, paxillin, vinculin and tensin. Protein kinases that colocalize with these structures include focal adhesion kinase (FAK), c-*src*, protein kinase C and integrin linked kinase (ILK) etc., (Yamada and Miyamoto, 1995; Dedhar and Hannigan, 1996).

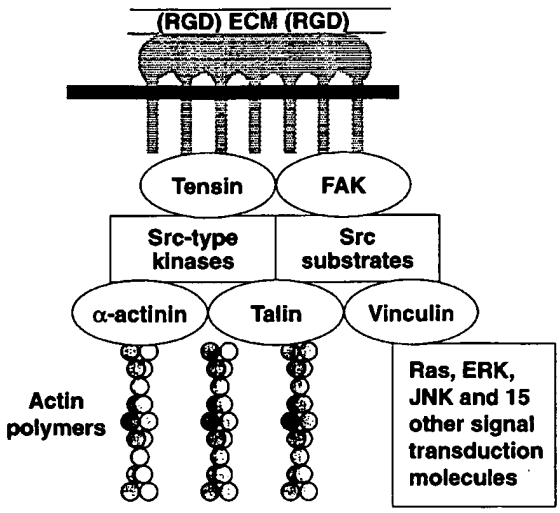


Figure 2 Model for the arrangements of different proteins in a focal adhesion complex. Arrangements of cytoskeletal proteins that colocalize to focal adhesion complexes *in vivo* and bind to integrins *in vitro* are shown. Ligand occupancy, integrin aggregation and tyrosine phosphorylation lead to the accumulation of F-actin and associated cytoskeletal proteins in a massive adhesive and signaling complex. ECM: Extracellular matrix, RGD: Arg-Gly-Asp, JNK: Jun Kinase

Focal adhesion kinase

Unlike growth factor receptors, integrins lack intrinsic tyrosine kinase activity. Yet, an early event during integrin signaling is the tyrosine phosphorylation of the non-receptor tyrosine kinase, FAK in response to cell adhesion (Schaller and Parsons, 1994; Richardson and Parsons, 1995; Parsons, 1996). All β_1 and α_v containing integrins share the ability to promote the assembly of focal adhesions and activate FAK. FAK is an unusual nonreceptor tyrosine kinase and lacks src homology (SH)2 and SH3 domains. The mechanism by which integrins activate FAK is incompletely understood. It is clear that this process is tightly coupled to the process of assembly of focal adhesion complexes. The phosphorylation of FAK is believed to initiate a cascade of phosphorylation events and new protein interactions required for adhesion-dependent signaling complexes. Talin binds directly to the carboxy-terminal domain of FAK (Chen *et al.*, 1995) and also interacts with vinculin and thereby paxilin (Brown *et al.*, 1996). Paxilin in turn binds to a distinct site in the carboxy terminus of FAK (Schaller *et al.*, 1995). Thus it is believed that the initial recruitment of FAK to activated integrins is indirect and mediated by talin. It is proposed that upon recruitment to focal adhesion complexes by talin, FAK undergoes conformational change and interacts through its amino terminal domain with the β subunit tail. The amino terminal domain of FAK plays a negative autoregulatory role, possibly by folding back onto the catalytic domain (Richardson and Parsons, 1996). Thus the conformational transition may be a prerequisite for FAK's catalytic activity. Integrin aggregation by ligand binding would then result in oligomerization of FAK and activation of FAK by trans-autophosphorylation. Autophosphorylation of FAK at residue 397 results in the binding of SH2 domain of Src and Fyn (Schaller *et al.*, 1994). The Src family kinases such as Src and Fyn then phosphorylate a number of FAK-associated proteins including paxilin, tensin and $p^{130\ CAS}$ (Vuori *et al.*, 1996; Schlaepfer *et al.*, 1997). Src can also phosphorylate FAK at tyrosine residue 925, creating a binding site for the Grb2-mSOS complex (Schlaepfer and Hunter, 1997). Finally autophosphorylated FAK can combine with and activate phosphatidyl-inositide-3 kinase (PI-3K) (Chen *et al.*, 1996). FAK is thus linked to a number of intracellular signaling pathways. The analysis of FAK knockout mice has provided important information on the biological function of FAK. Embryonic fibroblasts derived from these mice form numerous small focal contacts, but fail to form the large peripheral focal adhesions and these cells

migrate less efficiently than control cells (Ilic *et al.*, 1995). The observation that overexpression of FAK increases cell migration while a dominant negative form of the kinase inhibits it are also consistent with a role for this kinase in dynamic regulation of focal adhesions during cell migration (Cary *et al.*, 1996; Gilmore and Romer, 1996).

In contrast to FAK which is activated in response to cell adhesion, the activity of the serine/threonine-kinase termed integrin-linked kinase (ILK) appears to be inhibited in response to integrin ligation. ILK was originally isolated in the yeast two hybrid system by virtue of its ability to interact with the integrin $\beta 1$ subunit (Hannigan *et al.*, 1996; Dedhar and Hannigan, 1996). ILK also colocalizes to the focal adhesions like FAK. In contrast to FAK, ILK can be readily coimmunoprecipitated with various β integrins (Hannigan *et al.*, 1996; Radeva *et al.*, 1997). Overexpression of ILK in epithelial cells has been shown to induce anchorage-independent growth and malignant transformation (Radeva *et al.*, 1997). A third protein termed β_3 endonexin has also been identified from yeast two hybrid screens as a β_3 interacting protein (Shattil *et al.*, 1995). The physiological role of β_3 endonexin is currently unknown.

Role of Rho family of proteins in focal adhesion assembly

Alan Hall's group was the first to describe the assembly of focal adhesions and stress fibers in serum starved Swiss 3T3 cells. These cells retain few stress fibers and are devoid of focal adhesions and its components. Readdition of serum or LPA stimulates the formation of stress fibers and focal adhesion complexes within 2–5 min (Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes and Hall, 1995). Other growth factors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) insulin, bombesin also stimulate focal adhesion assembly, but with a longer time course. The induction of a variety of cortical actin assemblies can be ascribed to the action of individual Rho proteins. Rho family of proteins are RAS-related GTP binding proteins consisting of Rho A, B, C, D and E; Racs 1 and 2 and Rac E; and Cdc42, Rho G and TC10 (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Rho is involved in the organization of focal adhesions and stress fibers in response to LPA; Rac is directly responsible for the membrane ruffling and extension of lamellipodia and Cdc42 uniquely controls the formation of filipodia (Hall, 1994, 1998; Van Aelst and D'Souza-Schorey, 1997). There is evidence for the interconnection of these family members in a hierarchial manner: Cdc42 activates Rac and Rac activates Rho.

In addition to the growth factor receptors, Rho family members can also be activated by integrin ligation and clustering. Hotchin and Hall (1995) have demonstrated that the interaction of integrins with extracellular matrix is not sufficient to induce integrin clustering and focal complex formation, but requires the activity of Rho family members. The molecular basis for this regulation is not clear but may involve effector proteins which associate with and become activated by GTP-bound RhoA. Two lines of evidence support the notion that integrins can activate Rho. It has been known for some time that integrins can stimulate the production of Phosphatidyl Inositol biphosphate (PIP_2) and recent studies have provided evidence that this effect is mediated by Rho (Chong *et al.*, 1994; Hartwig *et al.*, 1995), possibly through its interaction with a type I isoform of phosphatidyl inositol 4-phosphate 5-kinase (PIP4-5K) (Ren *et al.*, 1996). The increase in PIP_2 synthesis by Rho A is potentially relevant to focal adhesion assembly because the actin binding activity of several cytoskeletal proteins such as profilin and gelsolin is modulated by PIP_2 *in vitro* (Hartwig *et al.*, 1995) and PIP_2 is enriched in focal adhesion plaques. In light of this, Gilmore and Burridge (1996) reported that the association of PIP_2 with vinculin induces a conformational change in vinculin, allowing it to interact with talin, which binds actin. Furthermore they showed that injection of anti-PIP_2 antibodies into fibroblasts inhibited LPA/Rho-induced stress fibers and focal adhesion formation, suggesting a role for PIP_2 in focal adhesion and stress fiber assembly. Second, dominant negative Rho has been shown to partially suppress the activation of ERK in response to ligation of integrins, suggesting that a Rho regulated pathway is necessary for the full activation of ERK following integrin mediated cell adhesion (Renshaw *et al.*, 1996).

How does activation of Rho A lead to recruitment of Focal adhesion proteins and assembly of focal adhesion? This question still remains unanswered completely. However, exciting progress has been made in recent years. The involvement of phosphoinositide kinases in Rho signaling has been reported. The effector proteins for Rho A include PIP4-5K, the recently identified serine/threonine kinase Rho-associated coil-coil containing protein kinase (ROCK) and protein kinase N which all associate with and are activated by RhoA (Amano *et al.*, 1997; Van Aelst and D'Souza-Schorey, 1997). In addition, the p190 Rho GTPase-activating protein (GAP) may also regulate integrin-clustering (Settleman *et al.*, 1992). Constitutive activation of p190 Rho-GAP disrupts integrin clustering and focal plaque formation. PI-3 kinase which phosphorylates PI(4) phosphate (PIP) or PI(4,5) biphosphate (PIP2) to generate PI (3,4)P_2 or PI (3,4,5)P_3 respectively has been shown to associate with integrin-associated focal adhesion complexes (Chen *et al.*, 1996). The SH2 domain of the p85 subunit of PI-3 K may be important for its association with tyrosine phosphorylated FAK. The role of PI3K in integrin induced changes in cell behaviour is not understood; however the evidence that inhibition of PI-3 K blocks growth factor induced actin arrangements suggests a possible involvement in integrin regulated cytoskeletal rearrangements.

Rho can also activate a serine/threonine protein kinase known as Rho Kinase, which plays an important role in the assembly of focal adhesions (Amano *et al.*, 1997). This kinase phosphorylates the myosin binding subunit (MBS) of myosin light chain phosphatase, thereby suppressing the activity of the enzyme (Kimura *et al.*, 1996). The resulting increase in myosin light chain phosphorylation is believed to induce a conformational change in myosin, thereby increasing its binding to actin filaments and promote actomyosin contractility and formation of focal complexes and stress fibers (Chirzanowska-Wondnicka

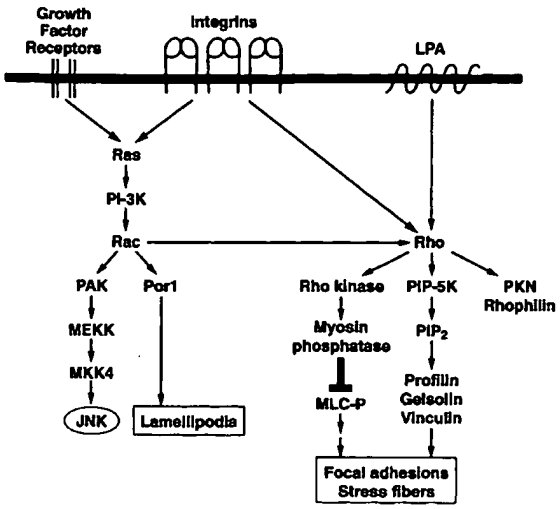


Figure 3 Signaling pathways involving integrin receptors and Rho family GTPases leading to focal adhesion complex and actin stress fiber formation. The integrins activate Rac via Ras and PI-3K and Rho through unknown mechanisms. Rac can also be activated by growth factors such as EGF, PDGF, and Insulin and Rho can be activated by LPA. Rac can also weakly activate Rho by an unknown mechanism. Rac and Rho interact with a number of target effectors. Some of these such as Porl, PIP-5Kinase and Rho kinase are responsible for organizing cytoskeletal structures such as lamellipodia and focal adhesions

et al., 1996). More recently, evidence has been presented that Rho kinase can also phosphorylate MLC at the same site phosphorylated by MLC kinase (Amano *et al.*, 1996). A model proposed for integrin-mediated and Rho-induced stress fiber formation is depicted in Figure 3.

In addition to the Rho family members, R-Ras, a GTP-binding protein homologous to Ha-Ras but with activities distinct from those of the transforming RAS proteins, has been shown to activate integrins. Expression of constitutively active R-Ras induced suspension cells to adhere to the extracellular matrix proteins by converting integrins from a low affinity to a high affinity state (Zhang *et al.*, 1996). Dominant negative R-Ras reduced the adhesiveness of cells suggesting that endogenous R-Ras can control integrin-ligand affinity states. The molecular basis for this regulation is not understood at the present time.

Role of integrins in cell-proliferation signaling pathways

In addition to growth factors and nutrients, many normal cells require adhesion to the extracellular matrix to proliferate. The adhesion-dependent activation of MAP kinase appears to be important in the pathway by which integrins regulate cell proliferation. A number of signaling proteins have been found to be associated with the focal adhesion complexes suggesting the involvement of integrin engagement in the activation of signaling pathways. It is now generally accepted that the activation of ERK in response to integrin ligation requires Ras signaling (Schlaepfer and Hunter, 1997; Wary *et al.*, 1996). Evidence is presented that certain integrins such as the laminin receptor $\alpha_6\beta_4$, the lamin/collagen receptor $\alpha_1\beta_1$, the fibronectin receptor $\alpha_5\beta_1$ and the vitronectin receptor $\alpha_v\beta_3$ are linked to the RAS-ERK signaling pathway by the adaptor protein Shc (Wary *et al.*, 1996; Giancotti, 1997). Shc is an SH2 and phosphotyrosine binding (PTB) domain adaptor protein which links tyrosine-phosphorylated signal transducers to Ras (Pawson, 1995). Upon recruitment to activated receptors, Shc is phosphorylated on tyrosine and binds to the Grb2-mSOS complex. This process results in the juxtaposition of the GTP exchange factor domain of mSOS to RAS, leading to its activation. The role of FAK in the activation of RAS-ERK pathway is not clearly established. Ligation of $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins which are all linked to Shc results in Erk activation, but ligation of other integrins does not produce this effect, even though FAK is stimulated (Wary *et al.*, 1996). A dominant negative mutant Shc was found to suppress ERK activation in response to integrin ligation (Wary *et al.*, 1996), but two distinct dominant-negative mutants of FAK do not (Lin *et al.*, 1997; Giancotti, 1997). These results indicate that Shc is necessary and sufficient to link specific integrins to the RAS-ERK signaling pathway. However, they do not exclude the possibility that FAK may cooperate with Shc to fully activate ERK in response to integrin ligation. As discussed above, integrin-mediated adhesion signals the autophosphorylation of FAK on Tyrosine 397, generating a binding site for SH2 domains of Src and related kinases (Calab *et al.*, 1995). The further phosphorylation of FAK at Tyr 925, presumably by Src generates a binding site for the SH2 domain of the adaptor protein GRB2 (Schlaepfer *et al.*, 1994). GRB2 signals through the RAS GDP/GTP exchange protein SOS, which has also been detected in the adhesion-dependent focal adhesion complexes containing FAK, GRB2 and c-Src (Yamada and Miyamoto, 1995) (Figure 4). The activation of RAS-ERK pathway by integrin ligation has been shown to require an intact cytoskeleton suggesting that the integrin-dependent cytoskeletal complexes are instrumental in the activation of the MAP kinase pathway.

MAP kinase in turn activates a number of transcription factors such as SRF, c-Myc that are involved in regulating growth and differentiation. Studies have shown that in primary cells, engagement of integrins linked to Shc activates transcription from the serum response element (SRE) and promotes progression through the G1 phase of the cell cycle in response to growth factors. Since growth factors also stimulate the RAS-MAP kinase pathway, it is likely that the integrins and growth factor receptors may synergize to enhance RAS-MAP kinase activation.

In addition to the participation of integrins in the activation of the RAS-ERK pathway via Shc, recent studies have shown that specific integrins may also cooperate with growth factor receptors directly. The $\alpha_v\beta_3$ integrin receptor has been found to uniquely associate with cytoplasmic signal transduction mediators of the insulin (and insulin like growth factor, (IGF) receptors (Vuori and Ruoslahti, 1994). One such signal transduction mediator is, insulin receptor substrate (IRS)-1. IRS-1 is tyrosine phosphorylated by the activated insulin (and IGF) receptor and as a result binds a number of other signaling molecules. In

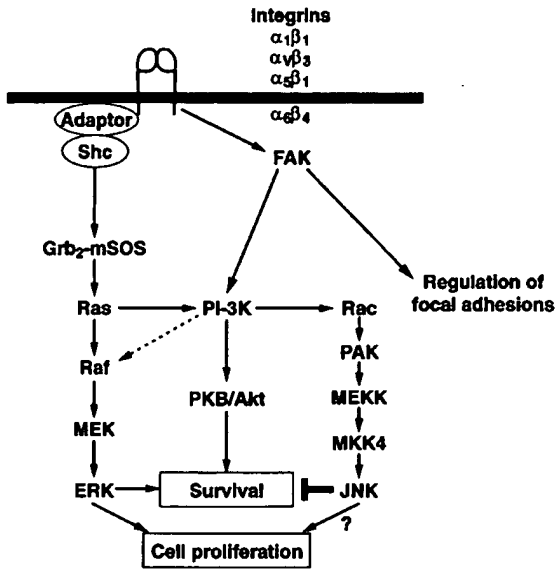


Figure 4 Integrin-mediated signaling pathways leading to cell proliferation and cell survival. Activation of Ras-ERK signaling pathway by integrins involves the recruitment of Shc via a transmembrane adaptor protein. Although all β_1 and α_v integrins can regulate focal adhesions via FAK, only some of them can recruit Shc and activate Ras-ERK signaling and promote cell cycle progression. Cell survival is promoted by integrins via Ras-ERK and Akt. Jnk may inhibit cell survival if ERK is not activated. There is also evidence for the involvement of PI-3K in integrin-mediated activation of Raf/ERK pathway. See the text for details

cells that have adhered to a substrate, such as vitronectin through the $\alpha_v\beta_3$ integrin, a subset of IRS-1 binds to the integrin. This interaction substantially enhances the growth stimulating effects of insulin and IGF. This interaction appears to be specific to integrin $\alpha_v\beta_3$ receptor. A 190 Kda protein that is phosphorylated in tyrosines as a result of PDGF receptor activity also binds to $\alpha_v\beta_3$ receptor, suggesting that there may be cooperation between this integrin and the PDGF pathway (Schneller *et al.*, 1997).

Regulation of cell cycle progression

Recent studies have indicated that cell adhesion is specifically required for the induction of cyclin D1 and for the activation of cyclin E-cdk2 complex in early-mid G1 phase (Fang *et al.*, 1996). Although cyclin D1 is regulated by cell adhesion at both the transcriptional and translational levels, the effect of cell adhesion on cyclin E-cdk2 activity appears to be indirect and mediated by downregulation of the cdk inhibitors p21 and p27 (Zhu *et al.*, 1996). These findings suggest several mechanisms by which integrin engagement could regulate the cell cycle.

Regulation of cell migration

Recently David Cheresh and coworkers have provided evidence that integrin mediated activation of ERKs influence the cells' motility machinery by phosphorylating and thereby enhancing myosin light chain kinase (MLCK) activity leading to phosphorylation of MLC (Klemke *et al.*, 1997). *In vitro* assays indicate that ERK can phosphorylate MLCK and ERK-phosphorylated MLCK displays an enhanced capacity to phosphorylate MLC in a calmodulin dependent manner. In summary, it is clear that growth factor receptors as well as integrin receptors influence the activity of one or more members of the MAP kinase family of signaling molecules. While MAP kinase signaling has been associated with the regulation of gene transcription events, David Cheresh's work clearly demonstrates that MAP kinase can promote cell migration on the ECM in a transcription-independent manner.

Role of integrins in cell survival signaling mechanisms

One important function of the integrin signaling pathways is to regulate anchorage dependence. Many mammalian cells are dependent on adhesion to the extracellular matrix for their continued survival. Early studies used fibroblasts which become growth inhibited in suspension, but remain viable. More recent studies have shown that certain specialized cell types such as endothelial cells and epithelial cells, when detached from substrate undergo apoptosis (Frisch and Francis, 1994; Meredith *et al.*, 1993; Frisch and Ruoslahti, 1997; Meredith and Schwartz, 1997). This phenomenon is referred to as 'anoikis' from the greek for homelessness. Anoikis is a mechanism to insure that cells which are displaced from their natural environment are eliminated. Tumor cells are characterized by their ability to grow in the absence of contacts with the extracellular matrix. Cell-detachment induced anoikis does not occur in cells expressing activated Src or Ras oncogenes. Hence, these oncogenes must therefore be able to provide a constitutive signal normally originating from integrin receptors.

The ability of the extracellular matrix to promote cell survival appears to be mediated by at least two distinct signaling pathways. Studies on the role of Shc in integrin signaling have indicated that in cells adhesion mediated by integrins not linked to Shc, results not only in cell cycle arrest, but also in apoptotic cell death (Wary *et al.*, 1996; Giancotti, 1997). In agreement with a critical role for Shc signaling in protection from apoptosis, a dominant negative form of Shc has been shown to induce apoptotic cell death in primary endothelial cells plated on fibronectin in the presence of mitogens (Wary *et al.*, 1996; Giancotti, 1997).

Role of phosphoinositide 3-Kinase and PKB/Akt in anoikis

Julian Downward's group studied the mechanism by which activated RAS oncogene protects MDCK epithelial cells from anoikis upon removal from extracellular matrix (Khwaja *et al.*, 1997). Their results suggest a following model of integrin signaling to the apoptotic machinery in untransformed epithelial cells. Cellular adhesion through integrins results in the activation of PI-3 kinase independently of signals from serum factors. The PI-3 kinase lipid products provide a protective signal acting through PKB/Akt which blocks entry into apoptosis. When epithelial cells are detached from the matrix, PI-3 kinase and PKB/Akt become

inactive even in the presence of serum factors and an apoptotic pathway is engaged. RAS activation leads to the constitutive activation of PI-3 kinase, PKB/Akt pathway accounting for the survival of RAS-transformed cells in the absence of adhesion to extracellular matrix. PI-3 kinase acting through PKB/Akt is therefore implicated as a key mediator of the aberrant survival of RAS-transformed epithelial cells in the absence of attachment. Interestingly, introduction of activated forms of the PI3 K p110 subunit, or of AKT rescued the MDCK cells from anoikis.

As with other signaling mechanisms, the PI-3 kinase and PKB/Akt pathway is also activated upon growth factor stimulation and mediates the cell survival mechanism in the absence of growth factor stimulation (Kennedy *et al.*, 1997; Marte and Downward, 1997). The mechanism by which integrin engagement leads to PI-3 kinase activation in normal cells is not known. One mechanism may be through FAK as this kinase has been found to associate with the SH2 domains of p85 of PI-3K, so it may contribute to the constitutive activation of PI-3 kinase. Alternatively, as PI3-K is a major target effector of RAS (Rodriguez-Viciana *et al.*, 1994), the activation of PI3-K by integrins could be mediated by the ability of Shc to activate RAS. Recent studies by King *et al.* (1997) have shown that integrin-mediated Erk-2, Mek-1 and Raf-1 activation but not Ras-GTP loading, was inhibited by PI-3K inhibitors demonstrating that PI-3K may function upstream of Raf-1 in integrin-mediated activation of Erk-2. Growth factor stimulation and integrin engagement leads to the stimulation of PI-3 kinase activity which results in the production of phosphatidyl inositol (3,4,5) triphosphate and Ptd Inositol (3,4)P_2 (Franke *et al.*, 1995). The binding of pleckstrin homology (PH) domain of PKB/Akt to these phosphoinositides both recruites PKB/Akt to the plasma membrane and in the case of PI(3,4)P2 directly stimulates its kinase activity (Franke *et al.*, 1997a, b). Full activation of PKB/Akt requires phosphorylation of Thr 308 by PDK1 and Ser 473by PDK2 (Alessi *et al.*, 1996, 1997). Activated PKB/Akt phosphorylates substrates resulting in a variety of biological effects including suppression of apoptosis. Figure 4 summarizes the integrin-mediated signaling pathways leading to cell proliferation and cell survival.

Role of cell shape in anoikis

Cell adhesion to matrix causes dramatic changes in cell shape. Therefore, the question arises, does the cytoskeletal organization and associated cell shape – as opposed to integrin signaling *per se* – suppress anoikis? Two series of studies specifically address this issue of cell shape and cell spreading in anoikis. Endothelial cells that were placed in suspension underwent anoikis. Addition of microbeads coated with RGD peptides that were too small to permit cell spreading did not rescue these cells. However, plastic surfaces coated with RGD peptides rescued cells from anoikis efficiently (Re *et al.*, 1994). These results suggest that mere attachment of cells through integrins is insufficient for rescue and that cell shape changes are required.

Recently in an interesting study the role of cell spreading was studied using a system in which the total surface area available for cell attachment was kept constant while forcing cells either to remain round or spread out (Chen *et al.*, 1997). This was accomplished by the use of microfabricated surfaces composed of islands of adhesive surface interspersed at varying distances from one another. When endothelial cells were cultured on these surfaces, round cells were found to be susceptible to apoptosis, whereas cells forced to stretch over widely dispersed small islands survived and proliferated (Chen *et al.*, 1997). These results again provide evidence that cell spreading following cell attachment is crucial to rescue cells from anoikis.

Different cells may differ in their ability to utilize specific integrins to activate the antiapoptotic mechanism. Recent studies using CHO cells engineered to express either $\alpha_5\beta_1$ or $\alpha_v\beta_1$ integrin as their fibronectin receptor have shown that only those cells expressing $\alpha_5\beta_1$ survived in serum free cultures (Zhang *et al.*, 1995). This response was associated with an elevated expression of the antiapoptotic protein Bcl-2. Other integrins such as $\alpha_v\beta_1$ or $\alpha_v\beta_3$ and various β_1 integrins fail to rescue cells from apoptosis under these conditions. It is possible that under other conditions cells depend on other integrins for survival (Brooks *et al.*, 1994; Boudreau *et al.*, 1995). Different cell types may differ in their integrin dependence under the same conditions. Thus vascular endothelial cells seem to depend on $\alpha_v\beta_3$ receptor for their survival (Brooks *et al.*, 1994). Inhibition of $\alpha_v\beta_3$ function during angiogenesis with anti-$\alpha_v\beta_3$ antibodies both inhibited proliferation and induced apoptosis, whereas treatment with antibodies that block β_1 integrin function did not (Brooks *et al.*, 1994). Similarly, prevention of interactions of mammary epithelium with the matrix by anti-β_1 integrin antibodies both inhibited proliferation and induced apoptosis (Boudreau *et al.*, 1995). These results suggest that the same integrin might connect to different pathways in different cell types and that a given integrin could provide a survival signal in one cell type and an anoikis signal in another. A possible physiological significance of the integrin-selective apoptosis phenomenon is that it may prevent cells from attaching to inappropriate places in the body, because attachment through the wrong integrin would induce apoptosis.

Role of integrins in cell transformation

It is clear from the above discussion that cells require both adhesion to immobilized matrix proteins and stimulation by serum or growth factors in order to proliferate. An important conclusion of these studies reviewed above is that signaling pathways activated by growth factor receptors and integrin receptors, ultimately converge to determine cell cycle progression. By contrast, tumor cell proliferation is generally independent of both adhesion and serum though anchorage independence is the feature that correlates best with tumorigenicity *in vivo* (Freedman and Shin, 1974). Proteins encoded by the oncogenes are generally components of the normal growth regulatory pathways that when overexpressed or mutated lead to irreversible activation of these pathways. Many oncogene encoded proteins also activate the integrin mediated pathways mainly through the Rho family proteins. Specifically, activated RAS has been shown to activate the cell

morphology pathway through RAC and recent studies have shown that activation of both the cell morphology pathway and ERK pathway are essential for transformation (Joneson *et al.*, 1996). Constitutive activation of both integrin-mediated and growth factor pathways would be necessary to elicit serum and anchorage-independent status to the transformed cells. Activation of a step on the growth factor portion only should lead to enhanced proliferation but not anchorage-independent growth. Thus fibroblasts transformed by Raf and constitutively active MEK grow very poorly in an anchorage-independent manner (Mansour *et al.*, 1994, and CC Kumar unpublished data). Activated Rac and Rho proteins have been shown to synergize with Raf to efficiently transform fibroblast cells, suggesting that Rac and Rho proteins are components of a separate pathway. Activation of a step on an integrin pathway prior to convergence would be expected to lead to anchorage-independent but serum-dependent growth. Thus oncogenes such as dbl and lbc which function as constitutively active guanine nucleotide exchange factors for the Rho family of proteins, induce anchorage-independent but serum-dependent growth (Schwartz *et al.*, 1996). lbc and dbl were identified by their ability to induce focus formation and tumorigenicity *in vivo* when expressed in 3T3 cells (Toksoz and Williams, 1994; Eva and Aaronson, 1985). Thus both lbc and dbl transformed cells show the same serum-dependence as control cells. These studies show that anchorage-independence and serum-independence can be separated. Understanding the mechanisms by which tumor cells gain the anchorage-independence status is an important area of investigation and may lead to the identification of novel targets for drug discovery.

Summary and therapeutic applications

An important conclusion from the studies reviewed above is that the signaling pathways activated by growth factor receptors and integrin receptors are extensively intertwined, most importantly at the level of Ras, PI-3K and FAK. In addition, certain integrins physically form complexes with growth factor receptors. Many signaling intermediates interact with and receive signals from various upstream elements and bind to a large number of downstream target effectors. Although much has been learned about integrin signaling, important questions remain concerning the circuitry responsible for the crosstalk among signals triggered by different integrins and integration of signals from integrins and growth factor receptors. Finally, an analysis of mechanisms by which tumor cells survive and proliferate in the absence of signals from the integrins will be an important area of investigation.

Research on integrin receptors has also led to a number of therapeutic applications and targets for tumor therapy. One integrin-directed drug, an anti-$\beta 3$ integrin antibody for the prevention of arterial restenosis, has reached the market place. Several other integrin based drugs such as the peptides containing the integrin-binding RGD sequence (Ruoslahti, 1996), and mimics of such peptides that specifically block individual integrins are also under development. These drugs target thrombosis ($\alpha_{IIb}\beta_3$ in platelets (Pierschbacher *et al.*, 1994), osteoporosis ($\alpha_v\beta_3$ in osteoclsts (Flores *et al.*, 1992)) and tumor-induced angiogenesis ($\alpha_v\beta_3$ in neovascular endothelial cells (Brooks *et al.*, 1994). In a recent exciting study, Ruoslahti's group used the *in vivo* selection of phage display libraries to isolate peptides that home specifically to tumor blood vessels. Two of these peptides – one containing α_v integrin-binding Arg-Gly-Asp motif and the other an Asn-Gly-Arg motif – were conjugated to the anticancer drug doxorubicin to demonstrate enhanced efficacy of the drug against human breast cancer xenografts in nude mice (Pasqualimi *et al.*, 1997). These peptide-drug conjugates exhibited significantly less toxicity, suggesting that it may be possible to develop targeted chemotherapeutic strategies based on the selective expression of integrin receptors in tumor vasculature.

In summary, recent studies on integrins have greatly increased our understanding of the central biological phenomena, such as anchorage-dependence, tumor-induced angiogenesis and tumor metastsis, as well as generated a number of possible approaches to new therapies for cancer.

Acknowledgements

I would like to thank Drs Martin McMahon and Lydia Armstrong for useful discussions and for their comments on the manuscript.

References

Alessi DR, Andjeikovic M, Caudwell FB, Morrice N and Norman DG. (1996). *EMBO J.*, **15**, 6541–6551.

Alessi DR, Kozlowski MT, Weng Q, Morrice N and Avruch J. (1997). *Curr. Biology*, **7**, 261–269.

Amano M, Chiharo K, Kimura K, Fukuta Y, Nakamura N, Matsura Y and Kaibuchi K. (1997). *Science*, **275**, 1308–1311.

Amano M, Ito M, Kimura K, Fukuta Y, Chihara K, Nakano T, Matsura Y and Kaibuchi K. (1996). *J. Biol. Chem.*, **271**, 20246–20249.

Bates RC, Buret A, Van Helden DF, Horton MA and Burns GF. (1994). *J. Cell Biol.*, **125**, 403–415.

Boudreau N, Sympson CJ, Werb Z and Bissel MJ. (1995). *Science*, **267**, 891–893.

Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu T, Klier G and Cheresh DA. (1994). *Cell*, **79**, 1157–1164.

Brown MC, Perrotta JA and Turner CE. (1996). *J. Cell Biol.*, **135**, 1109–1123.

Burridge K, Fath K, Kelly G, Nuckolls G and Turner C. (1998). *Ann. Rev. Cell Biol.*, **4**, 487–525.

Calab MB, Polte TR and Hanks SK. (1995). *Mol. Cell Biol.*, **15**, 954–963.

Cary LA, Chang JF and Guan JL. (1996). *J. Cell Biol.*, **109**, 1787–1794.

Chen HC, Appeddu PA, Isoda H and Guan JL. (1996). *J. Biol. Chem.*, **271**, 26329–26334.

Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD and Guan JL. (1995). *J. Biol. Chem.*, **270**, 16995–16999.
Chen CS, Mirksch M, Huang S, Whitesides GM and Ingber D. (1997). *Science*, **276**, 1425–1428.
Chirzanowska-Wondnicka M and Burridge K. (1996). *J. Cell Biol.*, **133**, 1403–1415.
Chong LD, Traynor-Kaplan A, Bokoch GM and Schwartz MA. (1994). **79**, 507–513.
Clark EA and Brugge JS. (1995). *Science*, **268**, 233–239.
Craig SW and Johnson RP. (1996). *Curr. Opin. Cell Biol.*, **8**, 74–85.
Dedhar S and Hannigan GE. (1996). *Curr. Opin. Cell Biol.*, **8**, 657–669.
Eva A and Aaronson SA. (1985). *Nature*, **316**, 273–275.
Fang F, Orend G, Watanabe N, Hunter T and Ruoslahti E. (1996). *Science*, **271**, 499–502.
Flores ME, Norgard M, Heinegard D, Reinholt F and Anderson G. (1992). *Exp. Cell Res.*, **201**, 526–530.
Franke TF, Kaplan DR and Cantley LC. (1997a). *Cell*, **88**, 435–437.
Franke TF, Kaplan DR, Cantley LC and Toker A. (1997b). *Science*, **275**, 665–668.
Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR and Tsichilis PN. (1995). *Cell*, **81**, 727–736.
Freedman VH and Shin S. (1974). *Cell*, **3**, 355–359.
Frisch SM and Francis H. (1994). *J. Cell Biol.*, **124**, 619–626.
Frisch SM and Ruoslahti E. (1997). *Curr. Opin. Cell Biol.*, **9**, 701–706.
Giancotti F. (1977). *Curr. Opin. Cell Biol.*, **9**, 691–700.
Gilmore AP and Burridge K. (1996). *Nature*, **381**, 531–535.
Gilmore AP and Romer LH. (1996). *Mol. Cell Biol.*, **7**, 1209–1224.
Hall A. (1994). *Ann. Rev. Cell Biol.*, **10**, 31–54.
Hall A. (1998). *Science*, **279**, 509–514.
Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolini MG, Radeva G, Filmus J, Bell JC and Dedhar S. (1996). *Nature*, **379**, 91–96.
Hartwig JH, Bokoch GM, Carpenter CL, Janmey LA, Taylor A, Toker A and Stossel TP. (1995). *Cell*, **82**, 643–653.
Hotchin NA and Hall A. (1995). *J. Cell Biol.*, **131**, 1857–1861.
Hynes RO. (1992). *Cell*, **69**, 11–25.
Hynes RO. (1994). *Bioessays*, **16**, 663–669.
Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T and Aizawa S. (1995). *Nature*, **377**, 539–544.
Joneson T, White MA, Wigler MH and Barsagi D. (1996). *Science*, **5250**, 810–812.
Juliano RL and Haskill S. (1993). *J. Cell Biol.*, **120**, 577–585.
Kennedy S, Wagner A, Conzen S, Jordan J, Bellacosa A, Tsichilis P and Hay N. (1997). *Genes Dev.*, **11**, 703–713.
Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne P and Downward J. (1997). *EMBO J.*, **16**, 2783–2794.
King WG, Mattaliano MD, Chan TO, Tsichilis PN and Brugge JS. (1997). *Mol. Cell Biol.*, **17**, 4406–4418.
Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A and Kaibuchi K. (1996). *Science*, **273**, 245–248.
Klemke RL, Cai S, Gianini AL, Gallagher PJ, de Lanerolle P and Cheresh D. (1997). *J. Cell Biol.*, **137**, 481–492.
Kolamus W and Seed B. (1997). *Curr. Opin. Cell Biol.*, **9**, 725–731.
Lin TH, Aplin AE, Shen Y, Chen Q, Schaller M, Romer L, Aukhil I and Juliano RL. (1997). *J. Cell Biol.*, **136**, 1385–1395.
Lo SH and Chen LB. (1994). *Cancer Metastasis Rev.*, **13**, 9–24.
Mansour SJ, Matten WT, Herman ASP, Candia JM, Rong S, Fukasawa K, Vande Woude G and Ahn NG. (1994). *Science*, **265**, 966–970.
Marte BM and Downward J. (1997). *TIBS*, **22**, 355–358.
Meredith JE and Fazeli B, Schwartz MA. (1993). *Mol. Biol. Cell*, **4**, 953–961.
Meredith JE and Schwartz M. (1997). *Trends Cell Biol.*, **7**, 146–150.
Nobes CD and Hall A. (1995). *Cell*, **81**, 53–62.
Parsons JT. (1996). *Curr. Opin. Cell Biol.*, **8**, 146–152.
Pasqualimi R, Koivnem E and Ruoslahti E. (1997). *Nature Biotech.*, **15**, 542–546.
Pawson T. (1995). *Nature*, **373**, 573–580.
Pierschbacher MD, Polarek JW, Craig WS, Tschopp JF, Sipes NJ and Harper JR. (1994). *J. Cell Biochem.*, **56**, 150–154.
Radeva G, Petrocelli T, Behend E, Leung-Hagesteijn C, Filmus J, Slingerland J and Dedhar S. (1997). *J. Biol. Chem.*, **272**, 13937–13944.
Re F, Zanetti A, Sironi M, Polentarutti E, Lanfrancone L, Dejana E and Cololta F. (1994). *J. Cell Biol.*, **127**, 537–546.
Ren XD, Bokoch GM, Traynor-Kaplan A, Jenkins GH, Anderson RA and Schwartz MA. (1996). *Mol. Cell Biol.*, **7**, 435–442.
Renshaw MW, Toksoz D and Schwartz MA. (1996). *J. Biol. Chem.*, **271**, 21691–21694.
Richardson A and Parsons JT. (1996). *Nature*, **380**, 538–540.
Richardson A and Parsons JT. (1995). *BioEssays*, **17**, 229–236.
Ridley A and Hall A. (1992). *Cell*, **70**, 389–399.
Ridley AJ, Paterson HF, Johnston CL, Diekmann D and Hall A. (1992). *Cell*, **40**, 401–410.
Rodriguez-Viciano P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J. (1994). *Nature*, **370**, 527–532.
Ruoslahti E. (1991). *J. Clin. Invest.*, **87**, 1–5.
Ruoslahti E. (1996). *Ann. Rev. Cell Biol.*, **12**, 697–715.
Ruoslahti E and Pierschbacher MD. (1987). *Science*, **238**, 491–497.
Ruoslahti E and Reed J. (1994). *Cell*, **77**, 477–478.
Schaller MD and Parsons JT. (1994). *Curr. Opin. Cell Biol.*, **6**, 705–710.
Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR and Parsons JT. (1994). *Mol. Cell Biol.*, **14**, 1680–1688.
Schaller MD, Otey CA, Hildebrand JD, Parsons JT. (1995). *J. Cell Biol.*, **130**, 1181–1187.
Schlaeper DD, Hanks SK, Hunter T and Van der Geer P. (1994). *Nature*, **372**, 786–791.
Schlaepfer DD and Hunter T. (1997). *J. Biol. Chem.*, **272**, 13189–13195.
Schlaepfer DD, Broome MA and Hunter T. (1997). *Mol. Cell Biol.*, **17**, 1702–1713.
Schneller M, Vuori K and Ruoslahti E. (1997). *EMBO J.*, **16**, 5600–5607.
Schwartz MA, Schaller MD and Ginsberg MH. (1995). *Ann Rev. Cell Biol.*, **11**, 549–600.
Schwartz MA, Toksoz D and Khosravi-Far R. (1996). *EMBO J.*, **15**, 6525–6530.
Settleman J, Narahimsan V, Foster LC and Weinberg RA. (1992). *Cell*, **69**, 539–549.
Shattil SJ, O'Toole T, Eigenthaller M, Thon V, Williams M, Babior BM and Ginsberg MH. (1995). *J. Cell Biol.*, **131**, 807–816.
Tamkun JW, Desimone DW, Fonda D, Patel RS, Buck C, Horwitz AF and Hynes RO. (1986). *Cell*, **46**, 271–282.
Toksoz D and Williams DA. (1994). *Oncogene*, **9**, 621–628.

Van Aelst L and D'Souza-Schorey C. (1997). *Genes and Dev.*, **11**, 2295–2322.
Vuori K and Ruoslahti E. (1994). *Science*, **266**, 1576–1578.
Vuori K, Hirai H, Aizawa S and Ruoslahti E. (1996). *Mol. Cell Biol.*, **16**, 2606–2613.
Wary KK, Mainiero F, Isakoff SJ, Marcanonio EE and Giancotti FG. (1996). *Cell*, **87**, 733–743.
Yamada KM and Miyamoto S. (1995). *Curr. Opin. Cell Biol.*, **7**, 681–689.
Zhang Z, Vuori K, Reed JC and Ruoslahti E. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 6161–6165.
Zhang Z, Vuori K, Wang M, Reed JC and Ruoslahti E. (1996). *Cell*, **85**, 61–69.
Zhu X, Ohtsubo M, Bohmer R, Roberts JM and Assoian RK. (1996). *J. Cell Biol.*, **133**, 391–403.

APPENDIX 8

OF
DECLARATION UNDER 37 C.F.R. § 1.132

DATED JANUARY 16, 2008

BY
DONALD GULLBERG

Content:

Gale et al., Proc Natl Acad Sci USA. 1996 Jan 9;93(1):357-61. (5 pages)

Berg et al., Genomics. 1999 Mar 1;56(2):169-78. (10 pages)

Laval et al., Biochim Biophys Acta. 1999 Nov 16;1435(1-2):61-70. (10 pages)

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 357–361, January 1996
Medical Sciences

Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*

CHERYL GALE*, DAVID FINKEL*, NIANJUN TAO*, MARILYN MEINKE*, MARK MCCLELLAN*, JENNIFER OLSON*, KATHLEEN KENDRICK†, AND MARGARET HOSTETTER*‡

*Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455; and †Department of Microbiology, Ohio State University, Columbus, OH 43210

Communicated by Mary Ellen Avery, Harvard Medical School, Boston, MA, September 26, 1995

ABSTRACT **The existence of integrin-like proteins in *Candida albicans* has been postulated because monoclonal antibodies to the leukocyte integrins αM and αX bind to blastospores and germ tubes, recognize a candidal surface protein of ≈185 kDa, and inhibit candidal adhesion to human epithelium. The gene *αINT1* was isolated from a library of *C. albicans* genomic DNA by screening with a cDNA probe from the transmembrane domain of human αM. The predicted polypeptide (αInt1p) of 188 kDa contains several motifs common to αM and αX: a putative I domain, two EF-hand divalent cation-binding sites, a transmembrane domain, and a cytoplasmic tail with a single tyrosine residue. An internal RGD tripeptide is also present. Binding of anti-peptide antibodies raised to potential extracellular domains of αInt1p confirms surface localization in *C. albicans* blastospores. By Southern blotting, *αINT1* is unique to *C. albicans*. Expression of *αINT1* under control of a galactose-inducible promoter led to the production of germ tubes in haploid *Saccharomyces cerevisiae* and in the corresponding *ste12* mutant. Germ tubes were not observed in haploid yeast transformed with vector alone, in transformants expressing a galactose-inducible gene from *Chlamydomonas*, or in transformants grown in the presence of glucose or raffinose. Transformants producing αInt1p bound an anti-αM monoclonal antibody and exhibited enhanced aggregation. Studies of αInt1p reveal novel roles for primitive integrin-like proteins in adhesion and in *STE12*-independent morphogenesis.**

The opportunistic pathogen *Candida albicans* is the leading cause of invasive fungal disease in neonates, diabetics, and immunocompromised patients and carries a high mortality despite prompt and appropriate anti-fungal therapy (1–3). Three important events in the pathogenesis of invasive candidal infection include adhesion to epithelium, penetration of epithelial barriers, and hematogenous dissemination. Complicating this cascade is the yeast's ability to transform from blastospores at the epithelial surface to elongated structures (germ tubes, pseudohyphae, and mycelia) that invade underlying tissues.

Several investigators have reported the existence of surface proteins in *C. albicans* that are antigenically, structurally, and functionally related to the α-subunits of the leukocyte integrins αM/β2 (Mac-1; CD11b/CD18) and αX/β2 (p150,95; CD11c/CD18) (4–11). Many monoclonal antibodies (mAbs) recognizing epitopes of αM or αX bind to blastospores or germ tubes of *C. albicans* (4–10). iC3b-coated sheep erythrocytes rosette with germ tubes of *C. albicans* (9), and the affinity constants for the binding of purified human iC3b to *C. albicans* or to leukocyte αM/β2 are virtually identical (5, 12). Environmental conditions such as increased temperature or glucose concentrations ≥10 mM augment not only the surface expression of this integrin-like protein (5, 11) but also the binding of iC3b (12). Recognition of ligands containing the tripeptide sequence arginine-glycine-aspartic acid (RGD) facilitates the adhesion of *C. albicans* to endothelial and epithelial cells (6, 11).

Among the leukocyte integrins, αM and αX share ≈70% sequence homology and considerable functional identity. These two α-subunits, together with αL, α1, and α2, contain an inserted or I domain of ≈200 amino acids that is involved in ligand binding (13–15). Located just C-terminal to the I domain in αM/αX are three divalent cation-binding sites; at the C terminus are a membrane-spanning region and a cytoplasmic tail, the latter containing a single tyrosine residue in αM and αX (13).

This manuscript reports the isolation of a *C. albicans* gene encoding a protein that shares these integrin motifs.§ Moreover, expression of the gene product in haploid *Saccharomyces cerevisiae* is associated with the production of germ tubes independently of Ste12p, a yeast transcription factor required for morphologic change in response to mating pheromones and nutrient limitations in *S. cerevisiae* (16). These results open the way for the discovery of other integrin-like proteins in primitive eukaryotes, for their study as precursors of vertebrate integrins, and for more detailed investigation of their roles in signal transduction and morphogenesis.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Reagents for Cloning. *C. albicans* 10261 (B311, serotype A) was purchased as a lyophilate (American Type Culture Collection). *Candida tropicalis* 7555 was isolated from the blood of a fungemic patient by the University of Minnesota Clinical Microbiology Laboratory. *S. cerevisiae* YPH500 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) is a galactose-utilizing strain obtained from the Yeast Genetic Stock Center (Berkeley, CA) (17). pBM272, an ARS/CEN-based yeast shuttle vector containing the *URA3* gene and the *S. cerevisiae GAL1-10* promoter (18), pGG201 containing a 990-bp open reading frame encoding a DNA-binding protein from *Chlamydomonas reinhardtii* (19), a 750-bp *Cla* I/*Hin*dIII fragment of the *C. albicans* actin gene, and *S. cerevisiae* strain M12B-T2 were gifts from James Bodley, Judith Berman, Paul Magee, and Beatrice Magee (all of the University of Minnesota), respectively. pSUL16, a gift from Judith Berman, contains the *S. cerevisiae STE12* gene interrupted with the yeast *LEU2* gene (20). *Escherichia coli* JM101, LE392, XL1-Blue-MRF', and pBluescript II SK(+) were obtained from Stratagene.

Cloning of *αINT1*. DNA from spheroplasts of *C. albicans* 10261 was isolated according to standard procedures (21), digested with *Sau*3AI, and packaged in λEMBL3 (Stratagene).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MM, minimal medium; mAb, monoclonal antibody.

‡To whom reprint requests should be addressed at: University of Minnesota, Department of Pediatrics, Box 296 UMHC, 420 Delaware Street, S.E., Minneapolis, MN 55455.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U35070).

Preliminary studies confirmed that a 3.5-kbp *Eco*RI fragment of *C. albicans* DNA hybridized with a 314-bp *Eco*RI/*Sma* I cDNA fragment derived from the transmembrane domain of human αM (kind gift of Dennis Hickstein, Veterans' Administration Medical Center, Seattle). A library enriched for 3.0- to 3.8-kbp *Eco*RI fragments was constructed by digestion of genomic DNA with *Eco*RI and ligation to pBluescript II SK(+). Primers for amplification of the *Eco*RI/*Sma* I αM cDNA fragment were as follows: upstream primer, 5'-GAATTCAATGCTACCCTCAA; downstream primer, 5'-CCCGGGGGACCCCCTTCACT. Plasmid minipreparations from a total of 200 colonies were screened by the sib selection technique for hybridization at 50°C with ^{32}P-labeled PCR product after confirmation of its nucleotide sequence (13). Five clones were isolated from three successive screenings. Two of the five clones gave reproducible signals after hybridization with a degenerate oligonucleotide encoding a conserved sequence (KVGFFK) in the cytoplasmic domain of αX (22): 5'-AA(AG) GT(CT) GG(AT) TT(CT) TT(CT) AA(AG)-3'. Both clones contained a 3.5-kbp *Eco*RI insert and failed to hybridize with a degenerate oligonucleotide from the *S. cerevisiae* gene *USO1* (23): 5'-GAA AT(ACT) GA(CT) GA(CT) TT(AG) ATG-3'. One of these clones (probe 2, Fig. 1*A*) was chosen for further analysis. A 500-bp *Hin*dIII subfragment (probe 3, Fig. 1*A*) was used to screen 20,000 clones from a library of *C. albicans*

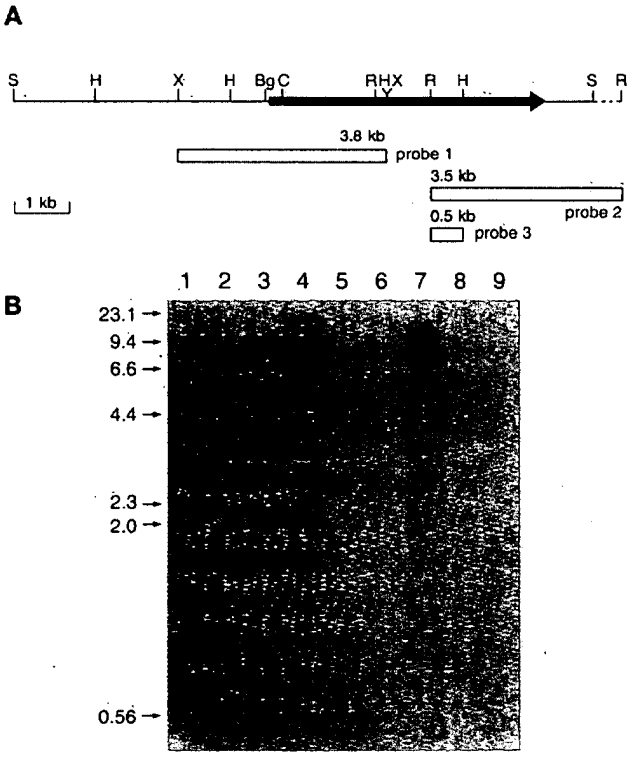


FIG. 1. (*A*) Restriction map of the 10.5-kbp *Sal* I genomic DNA fragment isolated from *C. albicans* 10261, with the open reading frame indicated by the bold arrow. Probe 1, 3.8-kbp *Xba* I fragment used for Southern and Northern blotting. S, *Sal* I; H, *Hin*dIII; X, *Xba* I; Bg, *Bgl* II; C, *Cla* I; R, *Eco*RI. (*B*) Southern blot of genomic DNA from *C. albicans* 10261 (lanes 1, 4, and 7), *C. tropicalis* 7555 (lanes 2, 5, and 8), and *S. cerevisiae* YPH500 (lanes 3, 6, and 9) digested with *Eco*RI (lanes 1–3), *Hin*dIII (lanes 4–6), and *Xba* I (lanes 7–9) and hybridized at high stringency with [α-^{32}P]dGTP-labeled probe 1 (hybridization at 65°C, final wash in 0.2× SSC/0.1% SDS at 65°C). The high molecular weight band (>12 kbp in lane 7) most likely represents incompletely digested DNA. Positions of *Hin*dIII-digested λDNA fragments are indicated on the far left. *Eco*RI and *Hin*dIII digests of four additional *S. cerevisiae* isolates from clinical and laboratory sources, as well as isolates of *Candida glabrata* and *Candida parapsilosis*, also failed to hybridize with probe 1.

10261 genomic DNA by the plaque hybridization technique (24). The largest hybridizing insert, a 10.5-kbp *Sal* I fragment (Fig. 1*A*), was isolated by agarose gel electrophoresis, cloned, and sequenced.

Sequence Analysis. Both strands of the 10.5-kbp *Sal* I fragment were sequenced by the method of gene walking on an Applied Biosystems model 373 automated sequencer in the University of Minnesota Microchemical Facility. Nucleotide and protein sequence analyses were performed with the Genetics Computer Group (University of Wisconsin, Madison) Sequence Analysis Software Package, version 7.0 (25).

Yeast Transformation and Gene Expression. The entire open reading frame of *αINT1* (*Bgl* II/*Sal* I fragment) was subcloned into pBM272 after digestion with *Bam*HI and *Sal* I, in order to place the *GAL1-10* promoter upstream of the *αINT1* start codon (pCG01). *S. cerevisiae* YPH500 was transformed with pBM272 or pCG01 by the lithium acetate procedure (26). Transformants were selected on agar-based minimal medium (MM = 0.17% yeast nitrogen base/0.5% ammonium sulfate) with 2% glucose, in the absence of uracil. Induction of *αINT1* was achieved by growing transformants containing pCG01 to mid-exponential phase in noninducing, nonrepressing medium (MM without uracil with 2% raffinose) at 30°C, then harvesting, washing, and resuspending them in inducing medium (MM without uracil with 2% galactose) at 30°C. YPH500 and YPH500 transformed with vector alone (pBM272) were grown under the identical conditions.

Southern and Northern Blotting. Genomic DNA and total RNA were isolated and electrophoresed by standard methods (27–30) and transferred to Hybond N+ nylon membranes (Amersham) by traditional capillary blotting.

Flow Cytometry. Anti-peptide antibodies were prepared in rabbits (Cocalico Biologicals, Reamstown, PA) to a 23-mer peptide encompassing the second divalent cation-binding site [amino acid (aa) 596–618] and to a 17-mer peptide spanning the RGD site and flanking residues (aa 1142–1158) in *αINT1*. The IgG fractions of preimmune and immune rabbit sera were isolated on protein A-Sepharose (Pharmacia). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) was used as the secondary antibody. For experiments with *S. cerevisiae* transformants, antibodies included OKM1 (anti-αM IgG2b) or MY9 as isotype control (Coulter) and FITC-conjugated goat anti-mouse IgM/IgG (Biosource International, Camarillo, CA) (7, 11).

Insertional Inactivation of *STE12* in *S. cerevisiae*. YPH500 was transformed with pSUL16 by standard techniques (26) and chromosomal integrants of the disrupted *STE12* gene were selected on leucine-deficient MM. After confirmation of sterility, *ste12* mutants were transformed with pCG01 as described above.

Aggregation Assay. The degree of aggregation of *C. albicans* and *S. cerevisiae* transformants was determined according to published methods (31).

RESULTS

Restriction Map and Southern Blotting. The restriction map of *αINT1* with its 5' and 3' flanking sequences is displayed in Fig. 1*A*. Fig. 1*B* shows that a 3.8-kbp *Xba* I probe from *αINT1* hybridized with *Eco*RI, *Hin*dIII, and *Xba* I fragments from *C. albicans* (lanes 1, 4, and 7) but not from *C. tropicalis* 7555 or *S. cerevisiae* YPH500. Among the yeast strains tested, this DNA fragment is unique to *C. albicans*.

Sequence Analysis of *αINT1*. Analysis of the nucleotide sequence revealed an open reading frame sufficient to encode a 1664-residue polypeptide with a theoretical molecular mass of 187,989 Da and no extensive homologies with other proteins. Fig. 2 compares the derived aa sequence of αInt1p with the characteristic motifs of several integrin α-subunits. BESTFIT

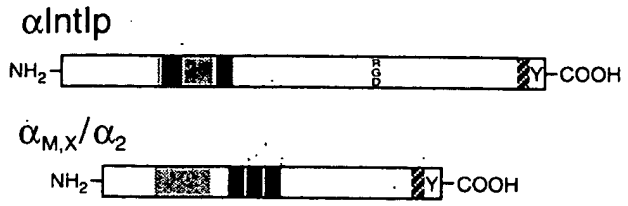


FIG. 2. Schematic diagram comparing the structures of αM, αX, and α2 with that of αIntlp. Gray regions represent the ligand-binding or I domain, the EF-hand divalent cation-binding motifs are indicated in black, and the transmembrane regions are hatched. RGD indicates the approximate location of this sequence in αIntlp (aa 1149–1151). The α-subunit schematic is modified from the sequence reported by Corbi *et al.* (13).

analysis (32) located a putative I domain at aa 230–470, with ≈18% identity to the I domain of human αM. Within this I-domain-like region are three potential partial MIDAS motifs (DXSX) for the coordination of divalent cations (33). This same region (aa 230–470) also displayed 25% identity to the nonrepeat region of the fibrinogen-binding protein of *Staphylococcus aureus* (34). Chou–Fasman analysis (35) indicated multiple α-helices, two of them bracketing the second of two possible EF-hand divalent cation-binding motifs (aa 283–295 and aa 601–613). Fig. 3 shows that the amino acid sequence of the second divalent cation-binding site from αIntlp differs from the EF-hand consensus sequence (36) at only one residue, a non-cation coordinating site. A hydrophobic sequence is located at aa 1592–1617 as determined by Kyte–Doolittle hydrophobicity plotting (37). Just C-terminal to this putative membrane-spanning region in αIntlp is a unique tyrosine residue, also present in the cytoplasmic tails of αM and αX (13, 22).

In the upstream sequence, a putative TATA box is located at −34 from the start codon. The coding sequence also displays 24 N-glycosylation sites, 6 cysteine residues, and the tripeptide sequence arginine-glycine-aspartic acid (RGD) (aa 1149–1151), a feature of many integrin ligands but not of integrins themselves.

Localization of αIntlp in *C. albicans* and *S. cerevisiae*. Polyclonal antibodies prepared against the second divalent cation-binding site and the RGD sequence and flanking residues in αIntlp recognized 64–82% of *C. albicans* blastospores, while preimmune IgG bound to only 0.5–1% of yeast cells ($P < 0.0001$) (Table 1). These results confirm that αIntlp is a surface protein in *C. albicans* and that the second cation-binding site and the RGD site are in the extracellular region of the polypeptide. In *S. cerevisiae*, the binding of the anti-αM mAb OKM1 was significantly greater in transformants expressing *αINT1* vs. transformants containing vector alone for percent yeasts fluorescing (19.0% vs. 6.2%; $P \leq$ 0.004) and for mean channel fluorescence (181.8 vs. 65.7; $P \leq$ 0.013). These results confirm that αIntlp is surface-borne in *S. cerevisiae* transformants and is recognized by an anti-integrin mAb.

A

1	2	3	4	5	6	7
(D)	X	(DNS)	(ILVFYW)	(DENSTG)	(DNQGHKR)	(GP)

8	9	10	11	12	13
(ILVMC)	(DENQTSGCA)	X	X	(DE)	(ILVMFYW)

B

	1	2	3	4	5	6	7	8	9	10	11	12	13
αIntlp $I_{283-295}$	N	N	N	N	S	K	N	V	S	D	M	D	S
αIntlp $II_{601-613}$	D	S	N	D	G	D	R	E	D	N	D	D	I

FIG. 3. Comparison of divalent cation-binding motifs. (*A*) Consensus sequence for the 13-residue EF-hand divalent cation-binding motif (36). (*B*) The N-terminal (I) and more distal cation-binding site (II) in αIntlp. The standard single letter code for aa residues is used. (. . .), Acceptable amino acids; {. . .}, unacceptable amino acids; X, any amino acid. Cation coordinating sites are indicated in boldface type.

Table 1. Percent yeasts fluorescing and mean channel fluorescence of *C. albicans* blastospores after incubation with anti-peptide antibodies

Antibody source	% yeasts fluorescing	Mean channel fluorescence
Control 12	1.0 ± 0.5	67.4 ± 24.6
UMN12	82.4 ± 8.6*	317.0 ± 24.7*
Control 13	0.40 ± 0.36	36.4 ± 9.2
UMN13	64.1 ± 2.3*	266.7 ± 9.2*

Values represent the mean ± SD of three experiments done on different days using different aliquots of *C. albicans* 10261. UMN12 is the antibody to the second divalent cation-binding motif and UMN13 is the antibody to the RGD region of αIntlp. Control 12 and 13 are preimmune IgGs from rabbits prior to immunization with UMN12 and UMN13, respectively. A one-tailed Student's *t* test was used for statistical calculations.
*$P < 0.0001$ vs. control in all comparisons.

Expression of *αINT1* in *C. albicans* and *S. cerevisiae*. Hybridization of probe 1 with total RNA isolated from *C. albicans* blastospores detected message of ≈5.5 kb (Fig. 4*A*). In *S. cerevisiae*, *αINT1* message was detected in pCG01 transformants 6 hr after induction with 2% galactose and continued to be expressed for at least 24 hr (Fig. 4*B*, lanes 1 and 3). As expected, message was not detected in pCG01 transformants grown under conditions of repression (Fig. 4*B*, lanes 2 and 4) or in pBM272 transformants (Fig. 4*B*, lanes 5 and 6).

Coincident with the detection of *αINT1* message, the majority of the pCG01 transformants formed elongated cell projections reminiscent of germ tubes (Fig. 5*A*). These structures continued to be present for 24 hr and could be detected at galactose concentrations ≥ 0.05%. pCG01 transformants exhibited polar budding, typical of haploid organisms, rather

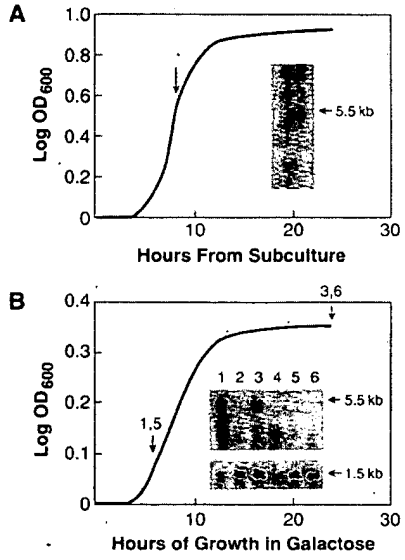


FIG. 4. (*A*) Northern blot of *C. albicans* 10261 total RNA isolated from blastospores in mid-exponential growth (arrow) in MM with 2% glucose and hybridized with probe 1 (see Fig. 1*A*). (*B*) Northern blot of total RNA from *S. cerevisiae* transformants: pCG01 transformants grown in galactose (lanes 1 and 3), pBM272 transformants grown in galactose (lanes 5 and 6), and pCG01 transformants grown to mid-exponential phase (lane 2) and to late exponential phase (lane 4) under conditions of repression (2% glucose). Probe 1 was used for hybridization. The diffuse signal at 2 kbp in lanes 2 and 4 represents nonspecific binding of the probe to the 18S ribosomal RNA band. The signal at 1.5 kbp represents actin transcript.

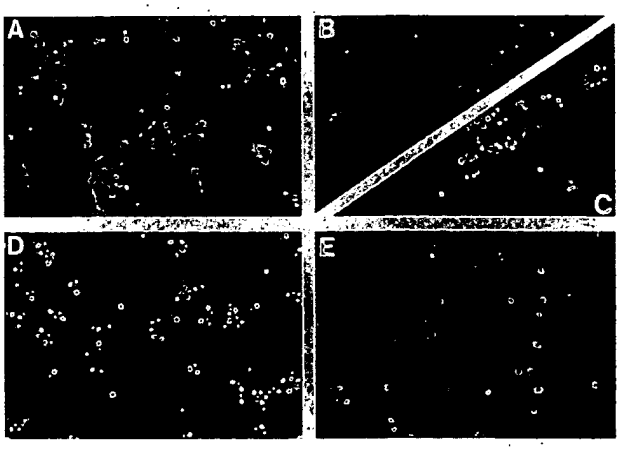


FIG. 5. Phase-contrast photomicrographs of *S. cerevisiae* transformants. pCG01 transformants (*A*) were grown to exponential phase in raffinose and then induced with 2% galactose for 6–24 hr. pBM272 transformants (vector without gene) (*B*), the parent strain YPH500 (*C*), *C. albicans* 10261 (*D*), and pGG201 transformants (galactose-inducible *C. reinhardtii* gene) (*E*) were grown identically. All yeast cells were photographed with a Leitz Wetzlar Laborlux 12 microscope equipped with a WILD MP551 Camera (Heerbrugg, Switzerland). (×500.)

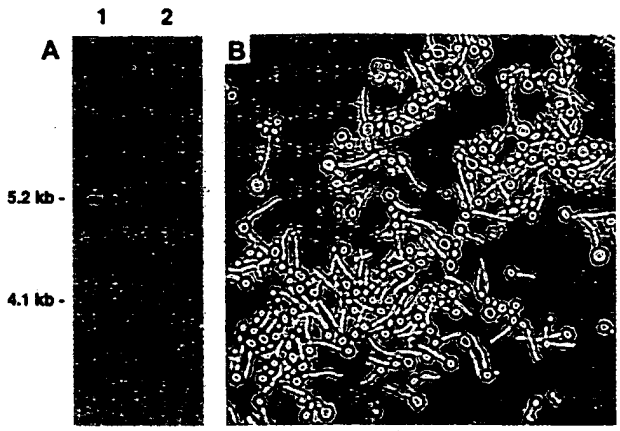


FIG. 6. (*A*) *Cla* I digests of genomic DNA from wild-type YPH500 (lane 1) and *ste12* mutant (lane 2). The blot was probed at high stringency with a 600-bp *Sph* I/*Cla* I fragment from pSUL16. (*B*) Phase-contrast photomicrograph of YPH500 *ste12* mutants transformed with pCG01 and grown in galactose for induction of *αINT1*.

than apical budding, which is typical of diploid organisms. pCG01 transformants (*MATα*) mated to a *MAT***a** yeast strain were able to form diploid organisms (data not shown).

pBM272 transformants, YPH500, and *C. albicans* 10261 did not form germ tubes when grown under the identical conditions (Fig. 5 *B–D*). pCG01 transformants did not exhibit germ tubes when grown in 2% raffinose, 2% glucose, or noninducing concentrations of galactose (0.02%) or when cured of the plasmid (data not shown). In addition, no germ tubes were observed with the galactose-induced expression of an ≈300-residue DNA-binding protein from *C. reinhardtii* (Fig. 5*E*). pCG01 transformants exhibited germ tubes during growth in liquid and on solid medium (MM with 2% galactose). Germ tubes were also observed in yeast strain M12B-T2 transformed with pCG01. Thus, the induction of germ tubes in haploid *S. cerevisiae* is specific to expression of *αINT1* from the plasmid pCG01.

Ability of Yeast Transformants to Aggregate. The aggregation index of pCG01 transformants equaled that of *C. albicans* germ tubes and significantly exceeded the aggregation index of *C. albicans* blastospores and *S. cerevisiae* pBM272 transformants (Table 2). This finding suggests that *S. cerevisiae* germ tubes synthesizing αInt1p are functionally similar to germ tubes in *C. albicans*.

Table 2. Percent aggregation of *C. albicans* and *S. cerevisiae* transformants

Yeast	% aggregation*
C. albicans	
Blastospores	62 ± 1
Hyphae	89 ± 4†
S. cerevisiae	
pBM272	65 ± 4
pCG01	80 ± 2‡

Values represent the mean ± SEM of four experiments, each done in triplicate. *C. albicans* blastospores were grown to mid-exponential phase in YPD medium (1% yeast extract/2% peptone/2% glucose) at 30°C. *C. albicans* hyphae were prepared by growth at 37°C in RPMI 1640 medium (GIBCO/BRL). *S. cerevisiae* pBM272 and pCG01 were grown in galactose-containing medium (see text).

*% aggregation = 100 × (OD_{540} final − OD_{540} initial)/OD_{540} final. A two-tailed Student's *t* test was used to determine statistical significance.

†$P = 0.0013$ vs. *C. albicans* blastospores.

‡$P = 0.026$ vs. *S. cerevisiae* pBM272.

Induction of Germ Tubes in *ste12* Mutants of *S. cerevisiae*. Insertional inactivation of *STE12* in YPH500 shifted the *Cla* I digestion fragment from 5.2 ± 0.1 kbp in the parent to 4.1 ± 0.1 kbp in the *ste12* mutant (Fig. 6*A*). The *Eco*RI fragment shifted from 10.5 ± 0.7 kbp (parent) to 5.0 ± 0.2 kbp (mutant). *ste12* mutants were unable to mate. After transformation of *ste12* mutants with pCG01 and induction of *αINT1* expression by growth in galactose, the mutants formed germ tubes (Fig. 6*B*). Therefore, the observed morphological change is independent of *STE12*.

DISCUSSION

We have isolated a gene encoding a putative integrin-like protein in *C. albicans* by screening a genomic library with conserved sequences from the transmembrane and cytoplasmic domains of human αM. αInt1p exhibits several motifs common to α-integrin subunits, including two EF-hand motifs and three partial MIDAS motifs within a putative I domain, a membrane-spanning domain, and a cytoplasmic tail with a conserved tyrosine residue at the C terminus. Because αM and αX recognize iC3b and fibrinogen as ligands (13), a 25% identity with the fibrinogen-binding protein of *S. aureus* (34) provides additional evidence for relationship.

Divalent cation-binding sites in the amino acid sequence of αM provided initial evidence of the leukocyte integrins' relationship to other vertebrate integrins (13). Both cation-binding motifs in αInt1p conform to the classic EF-hand consensus sequence. In comparison, two of the three cation-binding sites in αM agree at 11 of 13 residues; one of these and the third site require a gap to improve the alignment (13). Chou–Fasman analysis indicates that both divalent cation-binding sites in αInt1p, but not αM, are bracketed by α-helices, a conformation that facilitates cation binding (38). In addition, αInt1p contains three partial MIDAS motifs (DXSX) within the putative I domain. A full or partial MIDAS motif is present in all members of the I domain superfamily (15, 33). Of note, an I-domain-like sequence in *S. cerevisiae* Uso1p binds iC3b and the anti-αM mAb Mn41 (39) but has no divalent cation-binding sites or MIDAS motifs.

The presence of an I domain and an RGD sequence in the extracellular region of αInt1p should contribute to the adhesive capabilities of this protein. For example, an extracellular RGD sequence in the filamentous hemagglutinin of *Bordetella pertussis* facilitates adhesion of the bacterium to eukaryotic

cells (40). Another putative candidal adhesin is encoded by a 3.3-kbp genomic DNA fragment and enables transformed *S. cerevisiae* to adhere to polystyrene or buccal epithelial cells (41). However, its restriction map differs markedly from that of *αINT1*, and the nucleotide sequence has not been published.

In addition to a role as an adhesin, αInt1p leads to the production of germ tubes in haploid *S. cerevisiae* in a process independent of *STE12*. Although the morphological change correlates with expression of the candidal gene product and not with the production of other foreign proteins, we cannot discount the possibilities that αInt1p unnaturally disrupts the cytoskeletal architecture or the growth cycle or that other recognized morphogenic cascades, such as those involving the *CDC* genes (42, 43), may be implicated.

To date, only two genes that participate in morphogenesis in *C. albicans* have been reported. *ACPR*, also called *CPH1*, encodes a protein of 699 aa that is 74% identical to *S. cerevisiae* Ste12p (44, 45). *STE12* is an essential gene in at least two pathways involved in morphogenesis in *S. cerevisiae*: the induction of pseudohyphae in diploid cells on nitrogen-limited medium (46) and the invasive response of haploid cells on rich solid medium (47). Thus, the induction of germ tubes in *S. cerevisiae* transformants expressing *αINT1* after insertional inactivation of *STE12* suggests a novel pathway for integrin-mediated signaling. The second gene, *PHR1*, encodes an ≈580-aa polypeptide essential for pH-dependent morphogenesis in *C. albicans* (48). *ACPR* and *PHR1* encode intracellular regulatory proteins. The isolation of a gene encoding a surface-borne, integrin-like protein in *C. albicans* and its ability to induce morphological variants in haploid *S. cerevisiae* emphasize potential roles for *αINT1* in pathogenesis, signal transduction, and differentiation in *C. albicans* and *S. cerevisiae*.

C.G. is a St. Jude's Children's Research Hospital Fellow sponsored by the Pediatric Scientist Development Program. This research was also supported by funds from the National Institutes of Health (AI25827 and HD7031), the Pediatric AIDS Foundation, and the American Legion Heart Research Foundation to M.H.

1. Butler, K. M. & Baker, C. J. (1988) *Pediatr. Clin. North Am.* **35**, 343–563.
2. Rayfield, E. J., Ault, M. J., Keusch, G. T., Brothers, M. J., Nechemias, C. & Smith, H. (1982) *Am. J. Med.* **72**, 439–450.
3. Kiehn, T. E., Edwards, F. & Armstrong, D. (1980) *Am. J. Clin. Pathol.* **73**, 518–521.
4. Edwards, J. E., Jr., Gaither, T. A., O'Shea, J. J., Rotrosen, D., Lawley, T. J., Wright, S. A., Frank, M. M. & Green, I. (1986) *J. Immunol.* **137**, 3577–3583.
5. Gilmore, B. J., Retsinas, E. M., Lorenz, J. S. & Hostetter, M. K. (1988) *J. Infect. Dis.* **157**, 38–46.
6. Bendel, C. M. & Hostetter, M. K. (1993) *J. Clin. Invest.* **92**, 1840–1849.
7. Bendel, C. M., St. Sauver, J., Carlson, S. & Hostetter, M. K. (1995) *J. Infect. Dis.* **171**, 1760–1763.
8. Mayer, C. L., Diamond, R. D. & Edwards, J. E., Jr. (1990) *Infect. Immun.* **58**, 3765–3769.
9. Heidenreich, F. & Dierich, M. P. (1985) *Infect. Immun.* **50**, 598–600.
10. Hostetter, M. K., Lorenz, J. S., Preus, L. & Kendrick, K. E. (1990) *J. Infect. Dis.* **171**, 761–768.
11. Gustafson, K. S., Vercellotti, G. M., Bendel, C. M. & Hostetter, M. K. (1991) *J. Clin. Invest.* **87**, 1896–1902.
12. Gordon, D. L., Johnson, G. M. & Hostetter, M. K. (1987) *Immunology* **60**, 553–558.
13. Corbi, A. L., Kishimoto, T. K., Miller, L. J. & Springer, T. A. (1988) *J. Biol. Chem.* **25**, 12403–12411.
14. Hynes, R. O. (1992) *Cell* **69**, 11–25.
15. Colombatti, A., Bonaldo, P. & Doliana, R. (1993) *Matrix* **13**, 297–306.
16. Liu, H., Styles, C. A. & Fink, G. R. (1993) *Science* **262**, 1741–1744.
17. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122**, 19–27.
18. Johnston, M. & Davis, R. W. (1984) *Mol. Cell. Biol.* **4**, 1440–1448.
19. Konkel, L. M., Enomoto, S., Chamberlain, E. M., McCune-Zierath, P., Iyadurai, S. J. & Berman, J. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5558–5562.
20. Fields, S. & Herskowitz, I. (1987) *Mol. Cell. Biol.* **7**, 3818–3821.
21. Davis, R. W., Thomas, M., Cameron, J., St. John, T. P., Scherer, S. & Padgett, R. A. (1980) *Methods Enzymol.* **65**, 404–411.
22. Corbi, A. L., Miller, L. J., O'Connor, K., Larson, R. S. & Springer, T. A. (1987) *EMBO J.* **6**, 4023–4028.
23. Nakajima, H., Hirata, M. A., Ogawa, Y., Yonehara, T., Yoda, K. & Yamasaki, M. (1991) *J. Cell Biol.* **113**, 245–260.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed., pp. 108–125.
25. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
26. Ito, H., Jukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153**, 173–178.
27. Hoffman, C. S. & Winston, F. (1987) *Gene* **57**, 267–272.
28. Kohrer, K. & Domdey, H. (1991) *Methods Enzymol.* **194**, 398–401.
29. Southern, E. (1979) *Methods Enzymol.* **68**, 152–176.
30. Kaiser, C., Michaelis, S. & Mitchell, A. (1994) *Methods in Yeast Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 151–154.
31. Holmes, A. R., Cannon, R. D. & Shepherd, M. G. (1992) *Oral Microbiol. Immunol.* **7**, 32–37.
32. Smith, T. F. & Waterman, M. S. (1981) *Adv. Appl. Math.* **2**, 482–489.
33. Lee, J.-O., Rieu, P., Arnaout, M. A. & Liddington, R. (1995) *Cell* **80**, 631–638.
34. McDevitt, D., Francois, P., Baudaux, P. & Foster, T. J. (1994) *Mol. Microbiol.* **11**, 237–248.
35. Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* **47**, 45–147.
36. Bairoch, A. (1989) *PROSITE: A Dictionary of Protein Sites and Patterns* (Univ. of Geneva, Geneva).
37. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
38. Strynadka, N. C. & James, M. N. (1989) *Annu. Rev. Biochem.* **58**, 951–958.
39. Hostetter, M. K., Tao, N.-J., Gale, C., Herman, D. J., McClellan, M., Sharp, R. L. & Kendrick, K. E. (1995) *Biochem. Mol. Med.* **55**, 122–130.
40. Relman, D., Tuomanen, E., Falkow, S. L., Golenbock, D., Saukkonin, K. I. & Wright, S. (1990) *Cell* **61**, 1375–1382.
41. Barki, M. J., Koltin, Y., Yanko, M., Tamarkin, A. & Rosenberg, M. (1993) *J. Bacteriol.* **175**, 5683–5689.
42. Adams, A. E. M. & Pringle, J. R. (1984) *J. Cell Biol.* **98**, 934–945.
43. Hartwell, L. H. (1971) *Exp. Cell Res.* **69**, 265–276.
44. Malathi, K., Ganesan, K. & Datta, A. (1994) *J. Biol. Chem.* **269**, 22945–22951.
45. Liu, H., Kohler, J. & Fink, G. R. (1994) *Science* **266**, 1723–1726.
46. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992) *Cell* **68**, 1077–1090.
47. Roberts, R. L. & Fink, G. R. (1994) *Genes Dev.* **8**, 2974–2985.
48. Saporito-Irwin, S. M., Birse, C. E., Sypherd, P. S. & Fonzi, W. A. (1995) *Mol. Cell. Biol.* **15**, 601–613.

Genomics 56, 169–178 (1999)
Article ID geno.1998.5707, available online at http://www.idealibrary.com on IDEAL®

Cloning and Characterization of a Novel β Integrin-Related cDNA Coding for the Protein TIED ("Ten β Integrin EGF-like Repeat Domains") That Maps to Chromosome Band 13q33: A Divergent Stand-Alone Integrin Stalk Structure

Randal W. Berg,* Euphemia Leung,* Sheryl Gough,† Christine Morris,† Wei-Ping Yao,* Shu-Xia Wang,‡ Jian Ni,‡ and Geoffrey W. Krissansen*,[1]

*Department of Molecular Medicine, Faculty of Medicine and Health Science, The University of Auckland, Auckland, New Zealand; ‡Human Genome Sciences, Inc., 9410 Key West Avenue, Rockville, Maryland 20850; and †Cytogenetics and Molecular Oncology Unit, Christchurch School of Medicine, Christchurch, New Zealand

Received September 2, 1998; accepted December 7, 1998

Herein we describe the cDNA sequence of a novel human gene, *ITGBL1*, encoding a β integrin-related protein termed TIED [for ten β integrin epidermal growth factor (EGF)-like repeat domains]. Overlapping cDNA clones from fetal lung, HUVEC, and osteoblast cDNA libraries encode a sequence comprising a typical signal peptide, followed by a hydrophilic 471-amino-acid domain containing 10 tandem EGF-like repeats strikingly similar to those found in the cysteine-rich "stalk-like" structure of integrin β subunits. The EGF-like repeats of TIED and β integrins are unique in that they alternate in homology and possess two additional cysteines (eight in total) whose positions differ from those in the other eight-cysteine EGF-like domains of laminin, fibrillin, and the latent TGF-β binding proteins. TIED mRNA transcripts of 2.8 kb were detected in aorta, thymus, and osteogenic sarcoma cells. The *ITGBL1* gene was mapped to human chromosome 13, band 13q33. We suggest that *ITGBL1* may be linked in some way with the evolution of the integrin β subunits. © 1999 Academic Press

INTRODUCTION

Integrins are a superfamily of dimeric αβ cell-surface glycoproteins that mediate the adhesive functions of many cell types, enabling cells to interact with one another and with the extracellular matrix (ECM) (reviewed by Hynes, 1992). Electron microscopy reveals that integrins have a globular ligand-binding head composed of parts of both subunits and two stalks that extend to the plasma membrane (Carrell *et al.*, 1985; Nermut *et al.*, 1988). All eight identified integrin β subunits are highly similar (31–46% amino acid identity), where the stalk region is composed of a fourfold repeat of a cysteine-rich segment that is thought to be internally disulfide-bonded. No function has been ascribed to the stalk region, apart from the fact that it probably facilitates ligand binding by ensuring that the globular head extends beyond the glycocalyx. The stalk region appears to be a conduit for signaling events that either lead to integrin activation or are induced in response to ligand binding. Thus the AG89 mAb preferentially recognizes the cysteine repeat region following integrin activation and can itself induce activation of β1-integrin (Takagi *et al.*, 1997).

A previous comparison had revealed that the integrin β subunit cysteine-rich repeats were homologous with a cysteine-rich repeat region in domain III of laminin B chains (Yuan *et al.*, 1990). The four cysteine-rich repeats in β integrin subunits were most related to the first four repeats in domain III (20–40%). Part of the repeat unit of the laminin B1 chain was shown to contain a sequence similar to an EGF domain; however, the cysteine repeats in laminin are larger than those of EGF and contain eight rather than six cysteine residues (Pikkarainen *et al.*, 1988). Pairwise sequence identity comparisons between EGF modules of different proteins suggest that the laminin EGF repeats, and hence also the integrin repeats, are "outliers" and should be described as EGF-like until 3D structural comparisons can confirm their family membership (Campbell and Bork, 1993).

EGF-like domains contained in many growth factors, receptors, adhesion molecules, and proteins of the coagulation and fibrinolytic pathway have either been shown or are expected to participate in protein–protein or protein–cell interactions (Campbell and Bork, 1993; Appella *et al.*, 1988; Engels 1989). Interestingly, EGF domains in several proteins, including the integrin β5

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF072752.

[1] To whom correspondence should be addressed. Telephone: (64) 9 373-7599, Ext 6280. Fax: (64) 9 373 7492. E-mail: gw.krissansen@auckland.ac.nz.

0888-7543/99 $30.00

(Ramaswamy and Hemler, 1990) and β6 (Sheppard *et al.*, 1990) subunits, the laminin-associated protein nidogen (Timpl *et al.*, 1990), the glycoproteins PAS-6/7 (Andersen *et al.*, 1997) and lactadherin (Taylor *et al.*, 1997), and entactin (Dong *et al.*, 1995), contain the small tripeptide RGD, which is a major integrin binding site. Thus PAS-6/7 and lactadherin bind the integrin αvβ5 in an RGD-dependent fashion, and the RGD motifs in entactin bind αvβ3 and possibly α3β1. These EGF domains may participate in integrin-mediated RGD-dependent cell adhesion events. The site in laminin that mediates cell attachment, migration, and receptor binding was localized to the peptide CDPGYIGSR in the EGF-like repeat domain III of the B1 chain (Graf *et al.*, 1987). EGF domains in some ECM proteins are mitogenic as exemplified by those in the inner short arm structures of laminin (Panayotou *et al.*, 1989).

Here we report the cDNA sequence of a new member of the EGF-like protein family, termed TIED, that has the potential to provide novel insights into the evolution and alternative functions of the stalk structure of integrin β subunits.

MATERIALS AND METHODS

Cell culture. The human osteogenic sarcoma U-2OS cell line (Ponten and Saksela, 1967) obtained from the American Type Culture Collection (ATCC) was cultured in McCoy's 5A medium supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin, at 37°C in a 5% CO_2 atmosphere.

Screening of cDNA libraries. The TIED cDNA was initially identified as an expressed sequence tag (EST) following screens for integrin homology in an EST cDNA database using the BLAST network service provided by the National Center for Biotechnology Information. Partial-length TIED cDNA clones HSRAZ62 and HLHFV34 were identified in databases from human osteoclastoma and fetal lung cDNA libraries, respectively. Further clones were identified by screening fetal lung and umbilical vein endothelial cDNA libraries constructed using the LambdaZAP II vector (Stratagene, La Jolla, CA). Libraries were replica plated onto Gene Screen Plus filters (DuPont, Boston, MA), and screened as described previously (Yuan *et al.*, 1992) using either a 900-bp *Eco*RI/*Eco*RI fragment from clone HLHFV34 or a 223-bp PCR product, encompassing nucleotides 1216 to 1438, generated by PCR with the primers 62F 5′-ATGACGGAAGAACAAAGCAAGAA-3′ and 62R 5′-ATCCATCCCAGCAATCACAGTT-3′ from clone HSRAZ62.

DNA sequencing. DNA sequences were determined by cycle sequencing using an Applied Biosystems 373A automated DNA sequenator (The Centre for Gene Technology, School of Biological Sciences, University of Auckland, Auckland, New Zealand). The composite TIED sequence was obtained on both strands of the overlapping cDNA clones HSRAZ62, HLHFV34, S0003.9, HOHCH55, and HUVEC5.1.1, using a combination of Universal M13 and sequence-specific primers. Sequence analysis was performed using the Wisconsin package version 9.1 from the Genetics Computer Group (GCG) (Madison, WI).

Polymerase chain reaction (PCR). The expression of *ITGBL1* was analyzed by PCR using DNA templates from a human thymus cDNA library (ATCC) and cDNA prepared from mRNA extracted from U-2OS cells. Thermocycling parameters were 94°C for 1 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; followed by a final extension at 72°C for 3 min. For the chromosomal assignment, PCR was initially carried out with 24 cell hybrid DNAs in which 14 of the hybrids contained a single chromosome and the remaining 10 contained two to three chromosomes or one to three chromosomal fragments (Kelsell *et al.*, 1995). Subsequently a set of 7 cell hybrid DNAs was employed, in which cell hybrids MOG34A4, DUR4.3, SIR74ii, and LSR34S49 contain chromosome 13, and hybrids TWIN19-D12, CTP34B4, and DT1.2.4 together contain all other human chromosomes except for chromosomes 13, 9, and 19. Two primers, GM-F (5′-CGAATGAAATCCGAGTACCTATTAG-3′) and GM-R (5′-GCATCCCTGGCCTCTACCCAC-3′), were designed to amplify a region encompassing nucleotides 1618 to 1839 of the TIED cDNA sequence. They amplified a PCR product of 222 bp from human DNA, but not from mouse or hamster DNA. The PCR conditions for amplification from cell hybrid DNAs were as above except that annealing was carried out at 62°C and extension lasted for 45 s. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and transferred to GeneScreen Plus. Blots were hybridized with a ^{32}P-labeled 2.5 kb *Not*I/*Not*I fragment of clone HOHCH55 in 5× SSC, 5× Denhardt's solution, 50% formamide, with 1% SDS and 100 μg/ml denatured salmon sperm DNA, at 42°C. They were washed twice in 0.1× SSC, 0.1% SDS at 60°C for 30 min and autoradiographed.

Fluorescence in situ hybridization (FISH). Metaphase spreads were prepared from phytohemagluttinin-stimulated peripheral blood lymphocytes of a 46,XY male donor using standard cytogenetic procedures. The 2.5-kb insert of clone HOHCH55 was labeled with biotin-16-dUTP using a Biotin High Prime labelling kit (Boehringer Mannheim). Conditions for hybridization and immunofluorescent detection were essentially as described (Morris *et al.*, 1993), except that Cot 1 suppression was not required, slides were washed with 0.1× SSC at 60°C, and an additional amplification step was included. For precise chromosome band localization, DAPI and FITC images were captured separately for each metaphase from the fluorescence microscope using a Photometrics KAF1400 CCD camera and QUIPS

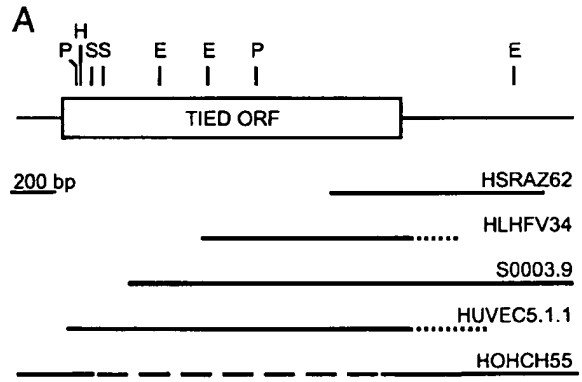


FIG. 1. Alignment of TIED cDNA clones, and their nucleotide and deduced amino acid sequences. **(A)** Partial restriction map of the composite TIED cDNA sequence and alignment of cDNA clones. The schematic at the top shows the open reading frame (ORF) as an open box, and 5′- and 3′-untranslated regions are shown as solid lines. The positions of recognition sites for the restriction enzymes *Pvu*II (P), *Hin*dIII (H), *Sma*I (S), and *Eco*RI (E) are indicated by vertical lines. The relative positioning of the TIED cDNA clones, which were sequenced on both strands, is indicated at the bottom with solid lines. The dashed region in HOHCH55 was sequenced on one strand only. The dotted 3′-untranslated regions in HLHFV34 and HUVEC5.1.1 denote sequence variation with corresponding regions in HSRAZ62, S0003.9, and HOHCH55. The scale bar indicates 200 bp. **(B)** Nucleotide and deduced amino acid sequences of the TIED molecule. The numbers in the left margin refer to nucleotide and amino acid positions. The first nucleotide of the start codon and the initiator methionine have each been assigned position 1. The 10 cysteine-rich integrin β- and EGF-like repeats are indicated with solid lines below the aa sequence, and the Cys residues in each repeat are numbered after Yuan *et al.* (1990). The stop codon is represented by an asterisk, the putative signal peptide and polyadenylation signal site (AATAAA) are underlined, and a potential site for N-linked glycosylation at position 405 is indicated (⊗).

B

```
-219                                                                ACCAGCACCCCGCCCAGAGCAGTGCCGCTGCCCAAATCC
-180 TCGCAGGCAGCTCATCAACGCAATTGCAACTCCGGCTGGAGCCCCGGACCTGCAAGCCTGGGTGTCCGTGGGTCCGTCTGCCCAGCCATC
-90  TGCTGGTGGCACCTCTCCCTCCTGCCGCCTCCCTCGGTGAACCCCACCTTGCAGAAGTGCAGCTCGCCCGGAGCAGCCCAGGAGCTCAGC
1    ATGCGTCCCCCAGGCTTCAGGAACTTCTTGCTGCTGGCGTCCTCCCTTCTCTTTGCTGGGTTGTCAGCTGTTCCTCAAAGCTTCTCGCCA
1    M  R  P  P  G  F  R  N  F  L  L  L  A  S  S  L  L  F  A  G  L  S  A  V  P  Q  S  F  S  P

91   TCTCTGAGGAGCTGGCCGGGCGCCGCCTGCAGGCTGTCCCGGGCCGAGTCCGAGCGACGCTGCCGCGCACCTGGGCAGCCCCCGGGGGCC
31   S  L  R  S  W  P  G  A  A  C  R  L  S  R  A  E  S  E  R  R  C  R  A  P  G  Q  P  P  G  A
                                                                    1————————————————————————
181  GCGCTGTGCCACGGCCGGGGCCGCTGCGACTGCGGCGTCTGCATCTGCCACGTGACTGAGCCGGGCATGTTCTTCGGGCCCCTGTGTGAG
61   A  L  C  H  G  R  G  R  C  D  C  G  V  C  I  C  H  V  T  E  P  G  M  F  F  G  P  L  C  E
     ———2————————————3———4———5———6————————————————————————————————————————————————7———
271  TGCCATGAGTGGGTGTGCGAGACCTACGACGGGAGCACCTGTGCAGGCCATGGTAAGTGTGACTGTGGCAAGTGCAAGTGTGACCAGGGA
91   C  H  E  W  V  C  E  T  Y  D  G  S  T  C  A  G  H  G  K  C  D  C  G  K  C  K  C  D  Q  G
     8         1————————————————————2————————————3———4————————5———6————————
361  TGGTATGGGGATGCTTGCCAGTACCCAACTAACTGTGACTTGACAAAGAAGAAAAGTAACCAAATGTGCAAGAATTCACAAGACATCATC
121  W  Y  G  D  A  C  Q  Y  P  T  N  C  D  L  T  K  K  K  S  N  Q  M  C  K  N  S  Q  D  I  I
     ——————————7———————————8                                        1————————————————————
451  TGCTCTAATGCAGGTACATGTCACTGTGGCAGGTGTAAGTGTGATAATTCAGATGGAAGTGGACTTGTGTATGGTAAATTTTGTGAGTGT
151  C  S  N  A  G  T  C  H  C  G  R  C  K  C  D  N  S  D  G  S  G  L  V  Y  G  K  F  C  E  C
     2————————————3———4———5———6————————————————————————————————————————————————7———8
541  GACGATAGAGAATGCATAGACGATGAAACAGAAGAAATATGTGGAGGCCATGGGAAGTGTTACTGTGGAAACTGCTACTGCAAGGCTGGT
181  D  D  R  E  C  I  D  D  E  T  E  E  I  C  G  G  H  G  K  C  Y  C  G  N  C  Y  C  K  A  G
               1————————————————————2————————————3———4————————5———6————————
631  TGGCATGGAGATAAATGTGAATTCCAGTGCGATATCACCCCCTGGGAAAGCAAGCGAAGATGCACGTCTCCAGATGGCAAAATCTGCAGT
211  W  H  G  D  K  C  E  F  Q  C  D  I  T  P  W  E  S  K  R  R  C  T  S  P  D  G  K  I  C  S
     ——————————7———————8                                        1————————————————————2———
721  AACAGAGGGACTTGTGTATGTGGTGAATGTACCTGTCACGATGTTGATCCGACTGGGGACTGGGGAGATATTCATGGGGACACCTGTGAA
241  N  R  G  T  C  V  C  G  E  C  T  C  H  D  V  D  P  T  G  D  W  G  D  I  H  G  D  T  C  E
     ——————————3———4———5———6————————————————————————————————————————————————7———
811  TGTGATGAGAGGGACTGTAGAGCTGTCTATGACCGATATTCTGATGACTTCTGTTCAGGTCATGGACAGTGTAATTGCGGAAGATGTGAC
271  C  D  E  R  D  C  R  A  V  Y  D  R  Y  S  D  D  F  C  S  G  H  G  Q  C  N  C  G  R  C  D
     8         1————————————————————2————————————3———4———5———
901  TGCAAAGCAGGCTGGTATGGGAAGAAGTGTGAGCACCCACAGTCCTGCACGCTGTCAGCTGAGGAGAGCATCAGGAAGTGCCAGGGAAGC
301  C  K  A  G  W  Y  G  K  K  C  E  H  P  Q  S  C  T  L  S  A  E  E  S  I  R  K  C  Q  G  S
     6————————————7———————8                                                  1————————
991  TCGGATCTGCCTTGCTCTGGGAGGGGTAAATGTGAATGTGGCAAATGCACCTGCTATCCTCCAGGAGATCGCCGGGTGTATGGCAAGACT
331  S  D  L  P  C  S  G  R  G  K  C  E  C  G  K  C  T  C  Y  P  P  G  D  R  R  V  Y  G  K  T
     ——————————2———————3———4———5———6————————————————————————————————————————————————
1081 TGTGAGTGTGATGATCGCCGCTGTGAAGACCTCGATGGTGTGGTCTGTGGAGGCCACGGCACATGTTCCTGTGGTCGCTGTGTTTGTGAG
361  C  E  C  D  D  R  R  C  E  D  L  D  G  V  V  C  G  G  H  G  T  C  S  C  G  R  C  V  C  E
     7———8         1————————————————————2————————————3———4———6———
1171 AGAGGATGGTTTGGAAAGCTCTGCCAACATCCGCGGAAGTGTAACATGACGGAAGAACAAAGCAAGAATCTGTGTGAATCAGCAGATGGC
391  R  G  W  F  G  K  L  C  Q  H  P  R  K  C  N  M  T  E  E  Q  S  K  N  L  C  E  S  A  D  G
     ——————————7———————8 ⊗                                  1————————————————————
1261 ATATTGTGCTCGGGGAAGGGTTCTTGTCATTGTGGGAAGTGCATTTGTTCTGCTGAAGAGTGGTATATTTCTGGGGAGTTCTGTGACTGT
421  I  L  C  S  G  K  G  S  C  H  C  G  K  C  I  C  S  A  E  E  W  Y  I  S  G  E  F  C  D  C
     ———2————————————3———4———5———6————————————————————————————————————————————————7———8
1351 GATGACAGAGACTGCGACAAACATGATGGTCTCATTTGTACAGGGAATGGAATATGTAGCTGTGGAAACTGTGAATGCTGGGATGGATGG
451  D  D  R  D  C  D  K  H  D  G  L  I  C  T  G  N  G  I  C  S  C  G  N  C  E  C  W  D  G  W
               1————————————————————2————————————3———4————————5———6————————
1441 AATGGAAATGCATGTGAAATCTGGCTTGGCTCAGAATATCCTTAACAATTACATGAGAGAGGTCTGGATTCTTATTTTTTCTGGGCCATT
481  N  G  N  A  C  E  I  W  L  G  S  E  Y  P  *
     ——————————7
1531 AGAACATATAAATGCGAAGGAAACCATGTATATTCACCACTAGGACAGGTTAAAAAGACCATTGTATGTTTTTCTATTTCTGAATTACGA
1621 ATGAAATCCGAGTACCTATTAGAAATGAGTTATGCAAATTTAGATGCAAATAACATTAGAAAAAAAAGATTCTTCCATAATTAACATAAG
1711 TGGTTCCTAACGAGAGCAATTTTTCCACCCAAAAGTCATTTGGCAACATCTACAGACAATTTTGATTGTCACACTGGGTCGGGTAGGAAG
1801 GTATGCTGCAGACATTTGGTGGGTAGAGGCCAGGGATGCTGCTGAGCATCCCGCAGTGTACAGGACAGCCCCCAAACAAGGAATTATCCA
1891 GCCCCAAATGCCAATAGGGCTCAAACTGAGAAACATTGAGTTATATGGCTATTAGAAATCCACATTCTTACACAAGAAAGACCATATTAG
1981 AATCTAAGGAAAACATGCATATTCACATTAATTAATCGATCAGATTTTTCCAGAATTCCGTATCAGTCACCATTTTAATATGGGGACAAT
2071 GAAGACAAGCACACAGGAGGTAGAATATCAGAGTGGGGCTGGATCAAGGGCAAAAACTGGTCATTAAGTCATCTGACATTAAATCATTTA
2161 GCCACTAAGTTATTTGTGTACTCTCACTTTAAACTCACCAAAGAAGATTCTCTTAAAGAAATTATGAAAAATGTACAATTTAACATTTTA
2251 AATAAATAGTGACAGAAGTTGTTTAAAAA
```

FIG. 1—*Continued*

(Vysis Inc., Downers Grove, IL) Smartcapture FISH software (version 1.3). QUIPS CGH/Karyotyping software (version 3.0.2) assisted in karyotype analysis.

Northern blot analysis. Human MTN I and II filters and a Human RNA Master Blot (Clontech) were screened with the ^{32}P-labeled 900-bp *Eco*RI/*Eco*RI fragment of the insert of clone HLHFV34. Hybridization was carried out at 60°C in ExpressHyb solution (Clontech). Filters were washed twice in 0.1× SSC, 1% SDS at 50°C for 30 min and autoradiographed. Total RNA was isolated from the osteogenic sarcoma cell line U-2OS as described (Chomczynski and Sacchi, 1987), separated on 1% agarose formaldehyde gels, and transferred to GeneScreen Plus. Blots were hybridized with the ^{32}P-labeled 2.5-kb insert of clone HOHCH55 in 5× SSC, 5× Denhardt's solution, 50% formamide, with 1% SDS and 100 μg/ml denatured salmon sperm DNA, at 42°C. They were washed twice in 0.1× SSC, 0.1% SDS at 50°C for 30 min and autoradiographed.

RESULTS AND DISCUSSION

Cloning of a Novel Integrin β Subunit-Related Molecule

A homology search (Altschul *et al.*, 1990) of a human EST cDNA database generated through the combined efforts of Human Genome Sciences, Inc. and The Institute for Genomic Research (Adams *et al.*, 1995; Feng *et al.*, 1996), using the known amino acid sequences of

integrin subunits, identified clones HSRAZ62 and HLHFV34 from osteoclastoma and fetal lung cDNA libraries, respectively, which represented a potential novel integrin β subunit. The HSRAZ62 clone was sequenced on both strands, and alignment of the translated sequence with integrin β subunit sequences revealed that it encoded two complete cysteine repeat domains highly similar to those contained in the β integrin stalk-like structure. However, no N-terminal methionine initiation codon was present, and the last cysteine repeat was not followed by a transmembrane domain, as in integrin β subunits. To isolate the full-length sequence for this unusual clone, a 223-bp HSRAZ62-derived PCR product (refer to Materials and Methods) was used to screen a variety of cDNA libraries including two prepared from human fetal lung and umbilical vein endothelial cells, from which positive clones were obtained. Clone S0003.9 from the fetal lung library and clone HUVEC5.1.1 from the endothelial cell library both extended the HSRAZ62 sequence, and a subsequent screen of the EST database identified the potential full-length cDNA clone HOHCH55 from an osteoblast cell cDNA library (Fig.1A).

Structure of the TIED ("Ten β Integrin EGF-like Repeat Domains") Molecule

The nucleotide and deduced amino acid sequence of the complete TIED molecule derived from the composite cDNA is shown in Fig. 1B. The 2493-nucleotide sequence includes 219 nucleotides of 5′-untranslated sequence, a 1485-nucleotide open reading frame encoding 494 amino acid residues, and 789 nucleotides of 3′-untranslated sequence that includes a consensus AATAAA poly(A) signal followed 18 nucleotides later by a poly(A) stretch. The presumptive methionine translational start codon is flanked by sequence that resembles but is not identical to a classical Kozak consensus, PurNNAUGPur. Nevertheless it is followed by a hydrophobic stretch of 23 amino acid residues that is typical of a signal peptide sequence (Fig. 2A). A recently submitted EST from the Washington University–NCI Human EST Project (Accession No. AA417383) extends the HOHCH55 sequence by 59 nucleotides and incorporates an in-frame stop codon, rendering it unlikely that the open reading frame extends upstream of the designated start codon. The putative signal peptide is followed by a predominantly hydrophilic domain of 471 amino acid residues, containing 10 EGF-like cysteine-rich repeats. The last repeat is incomplete, missing the C-terminal cysteine. The predicted molecular mass of an unglycosylated form of the mature protein is 51.4 kDa; however, there is one potential N-linked glycosylation site, Asn 405.

As this work was nearing completion, a BLAST search of the GenBank database revealed an entry, AB008375, whose sequence was essentially identical to that of TIED, except that it contains an extra G residue at nucleotide position 337, which alters the reading

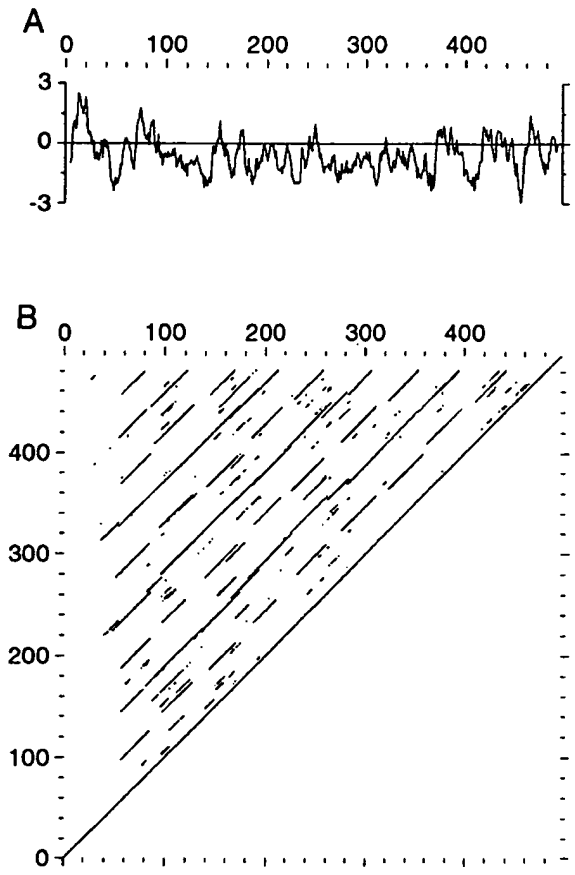


FIG. 2. Hydropathy and internal similarity plots of the TIED molecule. **(A)** Hydropathy plot (Kyte and Doolittle, 1982) of the deduced TIED protein, illustrating the hydrophobicity of the first 23 amino acid residues predicted to represent a functional signal peptide. **(B)** Dot-matrix comparison illustrating the repetitive nature of the deduced amino acid sequence of TIED. In this GCG plot, amino acid residues in the TIED sequence are compared with one another in pairwise fashion. Similarities are converted to dots that form clusters and diagonal lines, with complete identity along the central diagonal.

frame. Thus the encoded molecule is N-terminally truncated, being identical to TIED C-terminal to amino acid residue Gly 113, but extending only 21 residues further N-terminal. In addition to several nucleotide substitutions, AB008375 harbors a 68-bp deletion (nucleotides 145 to 212). The encoded molecule was proposed to be osteoblast-specific, but this seems unlikely given our expression data for TIED.

The TIED EGF-like Domains Are Remarkably Similar to Those of Integrin β Subunits

The repetitious modular structure of TIED is most clearly illustrated in Fig. 2B by a dot-matrix comparison, where the presence of repeats is visualized by lines and dashes that run parallel to the central diagonal that marks amino acid identity. Comparison of the deduced TIED sequence with EGF-like proteins in the GenBank database revealed that the TIED repeats were most similar with the β integrin cysteine-rich repeats. There are two features that distinguish the integrin β subunit and TIED repeats from the majority of other EGF-like proteins. The EGF domains of TIED

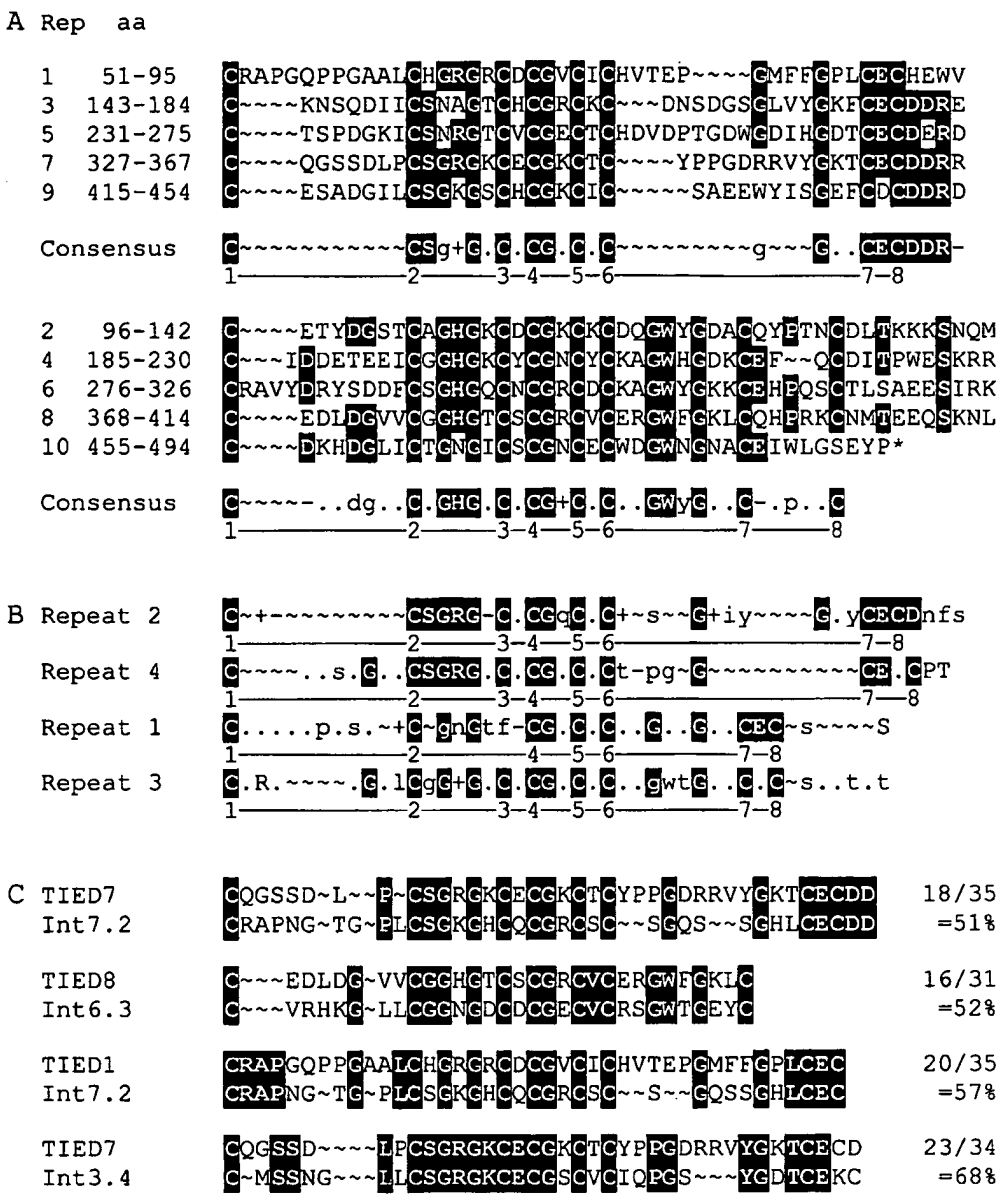


FIG. 3. The EGF-like domains of TIED are highly similar to those of β integrins. **(A)** The EGF-like domains of TIED alternate in homology. Amino acid sequences of the odd (upper) and even (lower) numbered EGF-like repeats in TIED are aligned, with consensus sequences shown below each group. Consensus amino acid residues are boxed in black. Numeration of amino acid residues is as in Fig. 1B. **(B)** The EGF-like domains of the β integrins also alternate in homology. Consensus sequences of the four EGF-like repeats of the human integrin β1 through β8 subunits have been aligned. Identical amino acid residues are boxed in black. **(C)** Similarity between the TIED and the β integrin EGF-like domains extends to large stretches of sequence identity. The first, seventh, and eighth EGF-like repeats of TIED have been aligned and compared with the second repeat of integrin β7 (Int7.2), the fourth repeat of integrin β3 (Int3.4), and the third repeat of integrin β6 (Int6.3), respectively. Identical amino acid residues are boxed in black. The percentage identity is indicated in the right margin. Gaps introduced to optimize the alignments are denoted by tildes (~). In the consensus sequences, uppercase letters indicate that 75 to 100% of the sequences aligned contain the amino acid at that position; lowercase letters indicate 50 to 75%; plus and minus signs indicate basic and acidic residues, respectively; and each dot represents the position of an amino acid residue that is not conserved. Conserved Cys residues are numbered as in Fig. 1B or after Yuan *et al.* (1990).

and β integrins contain eight cysteines, rather than the six cysteines found in the "classical" EGF domain (Figs. 3A and 3B). Only the first integrin repeat and the last TIED repeat are exceptions to the rule, possessing seven cysteines, as found in some of the EGF-like modules of fibrillin that have been termed "hybrid" domains (Corson *et al.*, 1993; Pereira *et al.*, 1993). Other members of the EGF-like family that possess eight-cysteine repeats include laminin (Pikkarainen *et al.*, 1988), fibrillin (Corson *et al.*, 1993; Pereira *et al.*, 1993), and the latent TGF-β binding proteins (LTBP) (Kanzaki *et al.*, 1990; Morén *et al.*, 1994; Tsuji *et al.*, 1990; Yin *et al.*, 1995). The positions of the "extra" two cysteine residues are unique to TIED and the β integrins and are not conserved in other eight-cysteine EGF-like domains present in fibrillin, laminin, and LTBPs (Fig. 4).

Second, the repeats show alternating similarity, such that odd-numbered repeats are most similar to one another, and vice versa, the even-numbered repeats are more similar to one another than they are to the odd-numbered repeats. To our knowledge this par-

```
EGF       CPLSHDGYCLHDGVCMYI~~EALDKYACNCVV~~~~~~GYIGERCQ

LamB1     CECHLQ~~GSVMAFCNPV~~~~~~TGQCHCFQ~~~~~~GVYARQCDRCLP~~GWWGFPSCQP

LTBP-2      CYR~~~SLGPGTCTLPLAQRITKQICCCSRVG~~~~KAWGSECEKCPLPGTEAFREIC
Fibn        CFL~~~RYEDEECTLPIAGRHRMDACCCSVGA~~~~AWGTEECEECPMRNTPEYEELC

TIED9       CES~~~~ADGILCS~~GKGSCHCGKCICS~~~AEEWYISGEFCDCDDRD
Int2.2      CRK~~~DNNSIICS~~GLGDCVCGQCLCHTSDVPGKLIYGQYCECDTIN
TIED6       CRAVYDRTSDDFCS~~GHGQCNCGRCDCK~~~~~~AGWYGKKCEHPQSCTLSAEESIRD
Int2.3      CER~~~~YNGQVCGGPGRGLCFCGKCRCH~~~~~~PGFEGSACQCERTTEG
```

FIG. 4. Comparison of the cysteine "footprint" of the TIED and β integrin EGF-like repeats with those in other proteins harboring eight cysteine EGF-like domains. The eight cysteines contained in representative EGF-like repeats found in the laminin B1 chain (LamB1), latent TGF-β binding protein (LTBP-2), and fibrillin (Fibn) have been aligned with cysteines in the sixth and ninth repeats of TIED and the second and third repeats of the integrin β2 subunit (Int2.2 and Int2.3, respectively). The eight cysteine-repeat motifs have been compared with the six-cysteine EGF repeat motif. Gaps introduced to optimize the alignments are denoted by tildes (~). Cysteine residues are boxed in black.

ticular feature is shared only by the β integrins and the TIED molecule. A schematic comparison of the TIED and β integrin structures is shown in Fig. 5. The odd-numbered TIED repeats are most similar to the even-numbered β integrin repeats. In particular the sequence CSGRG is highly conserved, as is the CECD sequence (except for the fourth integrin repeat) (Figs. 3A and 3B). The number of amino acids intervening between cysteines at positions 6 and 7 in these repeats varies markedly for both molecules. Vice versa, the even-numbered TIED repeats are most similar to the odd-numbered β integrin repeats, although this is not quite as obvious since the odd-numbered β integrin repeats appear to have diverged significantly during evolution. Importantly, the similarity between the β integrin and the TIED repeats does not relate just to conserved cysteine and glycine residues, but in some regions extends across the entire sequences. Comparison of the second repeat of the integrin β7 subunit with the first and seventh TIED repeats reveals 57 and 51% identity over 35 amino acid residues, respectively, which increases to 66% similarity when conservative substitutions are taken into account (Fig. 3C). Likewise, the fourth repeat of the integrin β3 subunit shares 68% amino acid identity over 34 amino acid residues with the seventh TIED repeat, and the third repeat of the integrin β6 subunit shares 52% amino acid identity with the eighth TIED repeat over 31 amino acid residues. Interestingly, EGF domains in several proteins contain the small tripeptide RGD, which is a major integrin binding site. The TIED sequence does not include an RGD motif or other common integrin binding motifs.

A class of EGF repeats found in functionally diverse proteins contain Ca^{2+} binding domains that have the consensus sequence Asp/Asn-x-Asp/Asn-Glu/Gln-x_n-Asp/Asn*-x_n-Phe/Tyr (where n is variable, and the asterisk indicates possible β-hydroxylation). Solution structures suggest that a conserved aromatic residue in a Gly-Aromatic-x-Gly motif between Cys 5 and 6 (Downing *et al.*, 1996; Rao *et al.*, 1995) and Ca^{2+} ions (Knott *et al.*, 1996) are both key elements involved in interdomain interactions that stabilize the three-dimensional structure of EGF modules. Some of the odd-numbered TIED repeats and the second β integrin repeat have the sequence Glu/Asp-x-Asp-Asp/Glu/Gln (where x is the eighth cysteine residue), resembling part of the core Ca^{2+} binding sequence.

Alternative Splicing of TIED 3'-Untranslated Regions

The 3'-ends of cDNA clones HUVEC5.1.1 and HLHFV34 diverge from clones HSRAZ62, S0003.9, and HOHCH55 at nucleotide positions 1476 and 1502, respectively (Fig. 6A). To determine whether the 3'-untranslated region might undergo alternative splicing, PCR primers were designed to the alternative 3'-untranslated regions and used to amplify TIED transcripts from fetal thymus cDNA. Primer pairs 62F and 57 (5'-TTTAACCTGTCCTAGTGGTG-3'; nucleotides 1565–1584) and 62F and 59 (5'-TGTCTGCAGCATACCTTCC-3'; nucleotides 1796–1814) that should amplify sequences contained in the S0003.9 and HOHCH55 cDNA clones both generated correct-sized PCR products, whereas the primer pair 62F/58 (5'-TAATGAATTCCAATGTCTGTGC-3') that should amplify

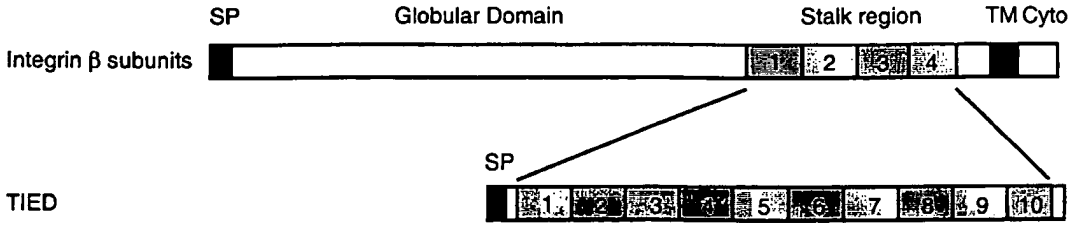


FIG. 5. Schematic comparison of the principal structural features of TIED with β integrins. The EGF-like repeats are numbered and shaded according to their alternating homology. Predominantly hydrophobic uncharged regions are denoted as solid blocks. SP, signal peptide; TM, transmembrane domain; and Cyto, cytoplasmic domain.

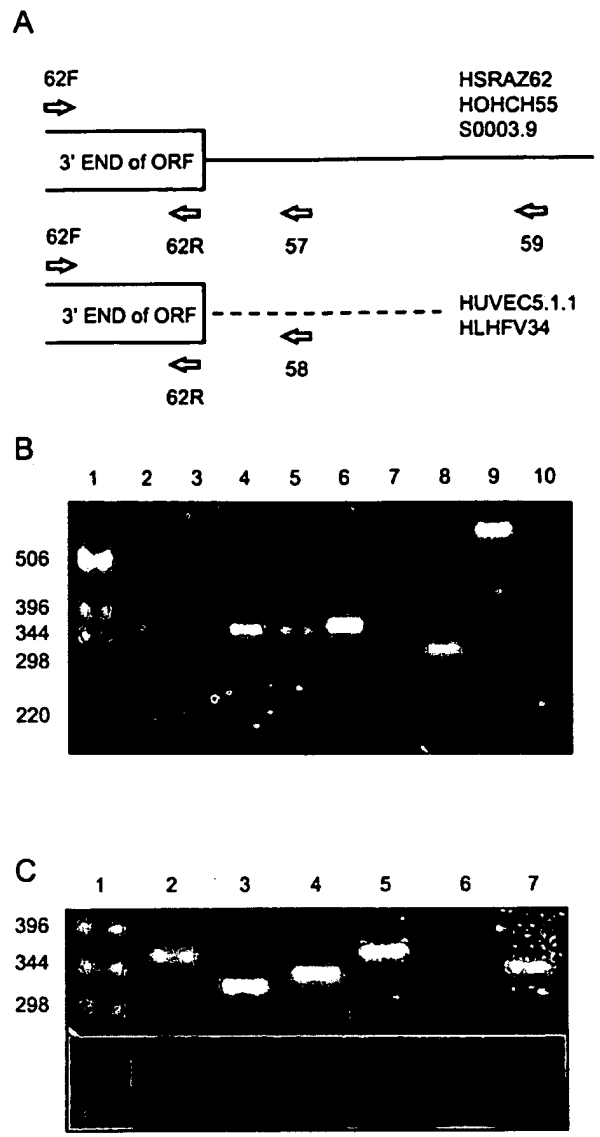


FIG. 6. TIED cDNA clones possess alternative 3′-untranslated regions: authenticating the 3′-ends by RT-PCR. To determine which of the different 3′-untranslated sequences in the various cDNA clones were authentic, RT-PCR analysis was performed using antisense primers to the alternative 3′-ends. (**A**) The locations of PCR primers are shown in the upper schematic diagram where the open reading frame (ORF) is boxed, and the 3′-untranslated region is denoted by solid or dashed lines. (**B**) PCR products obtained with primers 62F/62R (lanes **2** and **3**), 62F/57 (lanes **4** and **5**), 62F/58 (lanes **6**, **7**, and **8**), and 62F/59 (lanes **9** and **10**) were stained with ethidium bromide. Templates were plasmids containing the cDNA inserts HOHCH55 (lane **2**), S0003.9 (lanes **4** and **9**), HLHFV34 (lane **6**), and HUVEC5.1.1 (lane **8**), and cDNA from a fetal thymus library (ATCC) (lanes **3**, **5**, **7**, and **10**). A ladder of DNA markers is shown in lane **1**, with the sizes indicated in the left margin. (**C**) RT-PCR analysis of TIED transcripts in total RNA from human U-2OS osteogenic sarcoma cells (lanes **2** to **4**) and PCR from a HOHCH55 plasmid template (lanes **5** to **7**). PCR primers were 27/29 (lanes **2** and **5**), 62F/58 (lane **3** and **6**), and 62F/57 (lanes **4** and **7**). Lane **1**, DNA markers of 396, 344, and 298 bp. (**Top**) Ethidium bromide staining of PCR products; (**bottom**) the products have been hybridized to the ^{32}P-labeled insert of clone HOHCH55.

HUVEC5.1.1 and HLHFV34 sequences failed to generate a PCR product, despite producing the expected 333- and 379-bp products from the respective plasmid templates (Fig. 6B).

Since the HSRAZ62 and HOHCH55 clones were from osteoclastoma and osteoblast cDNA libraries, we examined expression of the alternative TIED transcripts in human osteogenic sarcoma U-2OS cells. RT-PCR with the primer pair 27 (5′-CTGTGGAAACTGCTACTGC-3′) and 29 (5′-CGTGCAGGACTGTGGGTGC-3′) and primer pair 62F/57 expected to amplify regions encompassing nucleotides 603 to 951 and 1216 to 1584 generated products of the expected sizes of 349 and 369 bp, respectively, which hybridized with a HO-

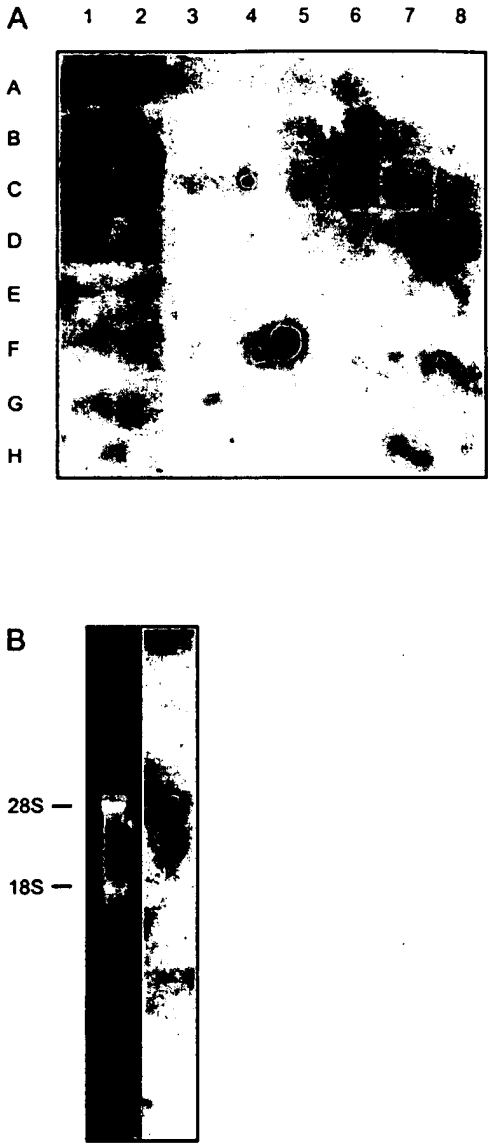


FIG. 7. Expression of TIED mRNA transcripts in various human tissues and in U-2OS osteogenic sarcoma cells. (**A**) A human RNA Master Blot (Clontech) was hybridized with a ^{32}P-labeled 900-bp *Eco*RI/*Eco*RI fragment from cDNA clone HLHFV34. The entire blot is shown illustrating detectable expression only in aorta (**C2**), whereas signals from dots containing poly(A)$^+$ RNA from 49 other tissues were not above background. The blot was rescreened to distinguish background spots from positive signals. Only the signal from aorta poly(A)$^+$ RNA was reproduced (not shown). (**B**) Northern blot of 15 μg of total RNA from U-2OS cells hybridized with the ^{32}P-labeled insert of clone HOHCH55 (right lane). The left lane illustrates an ethidium bromide stained agarose gel containing the total RNA isolated from U-2OS cells. Positions of 28S and 18S rRNAs are indicated in the left margin.

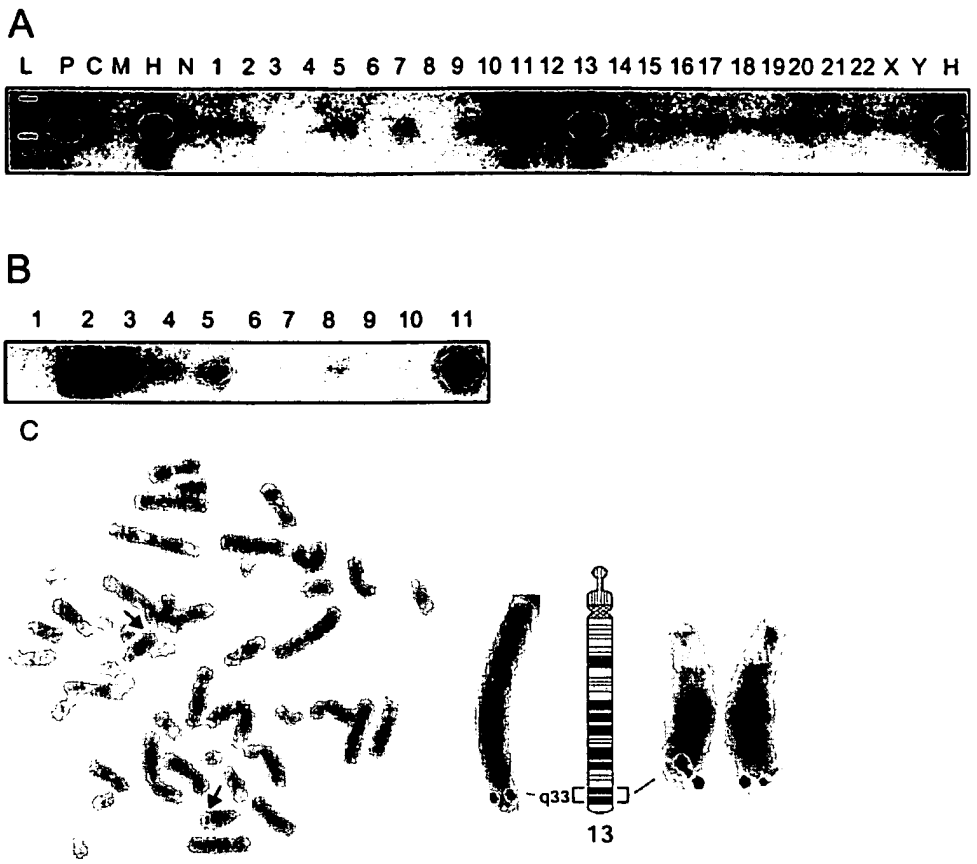


FIG. 8. The human *ITGBL1* gene maps to chromosome 13, band 13q33. (**A**) PCR analysis of a monochromosomal panel of human–rodent cell hybrid DNAs with the GM-F/GM-R primer pair generated a prominent 222-bp PCR product from chromosome 13, human DNA, and the HOHCH55 plasmid control. An autoradiograph of the PCR products hybridized with the ^{32}P-labeled insert of clone HOHCH55 is shown. Lanes correspond to DNA markers of 300 and 200 bp (**L**); plasmid HOHCH55 control (**P**); hamster (**C**), mouse (**M**), human (**H**) genomic DNA; no DNA control (**N**); and chromosome-specific somatic cell hybrid DNAs (chromosomes **1** to **22**, **X**, and **Y**). (**B**) PCR analysis of a second panel of human–rodent cell hybrid DNAs. PCR amplification with the GM-F/GM-R primer set was from DNAs of cell hybrids that contained chromosome 13 (MOG34A4, lane **2**; DUR4.3, lane **3**; SIR74ii, lane **4**; LSR34S49, lane **5**) and from hybrids that contained all other human chromosomes except for chromosomes 13, 9, and 19 (TWIN19-D12, lane **6**; CTP34B4, lane **7**; DT1.2.4, lane **8**). Control lanes include the following: lane **1**, no DNA; lane **9**, chromosome 9-specific hybrid DNA; lane **10**, chromosome 19-specific hybrid DNA; and lane **11**, chromosome 13-specific hybrid DNA. (**C**) Localization of the *ITGBL1* gene by FISH. Gray-scale inverted image of a complete metaphase cell showing fluorescent signals on chromosome 13, band q33, after hybridization of the biotinylated HOHCH55 cDNA probe (**left**). Idiogram of chromosome 13 with the q33 band bracketed and aligned with signals from enlarged copies of chromosome 13 selected from three different metaphase cells (**right**).

HCH55 cDNA probe (Fig. 6C). A 333-bp PCR product could also be amplified from U-2OS cDNA using the 62F/58 primer pair. Thus the 3′-ends of all the cDNA clones are authentic and result from alternative splicing, where those of HSRAZ62, S0003.9, and HOHCH55 are expressed in both thymus and osteogenic sarcoma cells, and those of the HUVEC5.1.1 and HLHFV34 are expressed only in the latter.

TIED mRNA Is Widely Expressed but Not Abundant

TIED cDNA clones were detected in osteoclastoma, osteoblast, umbilical vein, and fetal lung cDNA libraries, suggesting that TIED might be widely expressed; however, no clones were obtained from fetal heart and adrenal gland tumor cell derived libraries. Screening of a human RNA master blot (Clontech) containing RNAs from 50 different tissues revealed readily detectable expression of TIED mRNA transcripts only in aorta (Fig. 7A), suggesting that the TIED message is not particularly abundant in the tissues examined apart from aorta. TIED transcripts were not detected in either adult or fetal heart, suggesting that expression was specific for aorta. Northern blot analysis of total RNA prepared from U-2OS osteogenic sarcoma cells revealed a single transcript of approximately 2.8 kb (Fig. 7B).

The Human ITGBL1 Gene Maps to Chromosome 13, Band 13q33

PCR from genomic DNA of a panel of human–rodent hybrid cell lines was used to map the human *ITGBL1* gene to a particular chromosome. The expected 222-bp PCR product was specifically amplified from human genomic DNA and from the 289 hybrid, which contains human chromosome 13 and fragments of chromosome 8, 11, and 12 (Fig. 8A). *ITGBL1* sequences were not amplified from hybrids C4A, JICl4, and 1aA9602+, which contain human chromosomes 8, 11, and 12. As-

signment of the *ITGBL1* gene to chromosome 13 was confirmed by PCR analysis of a second series of chromosomal hybrids. An *ITGBL1* PCR product was amplified from the DNA of four hybrids that contained chromosome 13 (MOG34A4, DUR4.3, SIR74ii, and LSR34S49), but not from hybrid DNAs that contained all other human chromosomes except for chromosomes 13, 9, and 19 (TWIN19-D12, CTP34B4, and DT1.2.4) (Fig. 8B).

The precise localization of the *ITGBL1* gene was determined by FISH analysis using the HOHCH55 cDNA insert as a probe. Of 40 metaphase cells examined, 40 showed fluorescent signals on one or both chromosomes 13, specifically across band q33 (Fig. 8C). No additional site-specific signals were detected on any other chromosome. Other genes that have been mapped to chromosome band 13q33 include the pro alpha 1 and 2 (IV) collagen genes (Boyd *et al.*, 1988), the DNA ligase IV gene (Wei *et al.*, 1995), and the gene for xeroderma pigmentosum complementation group G (XPG) (Samec *et al.*, 1994). In terms of disease association, band 13q33 is a site for integration by human papilloma virus-33 (Gilles *et al.*, 1996); it is amplified in oral squamous cell carcinomas (Matsumura, 1995) and is commonly deleted in ovarian cancer (Yang-Feng *et al.*, 1992).

In summary, we predict that TIED is a secreted protein linked in evolution to the stalk-like structure of integrin β subunits. Whether an ancestral TIED-like molecule was integrated into β integrins via gene conversion and attributes integrins with novel functions is not known. Given that EGF-like domains participate in protein–protein and protein–cell interactions, future studies will need to appraise whether TIED protein has proadhesive, anti-adhesive, and/or growth factor activities.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Wellcome Trust (UK), the Health Research Council of New Zealand, The Royal Society of New Zealand, the Lottery Grants Board of New Zealand, the Cancer Society of New Zealand Inc., the Marsden Fund, the Auckland Medical Research Foundation, the Maurice and Phyllis Paykel Trust, and the Multiple Sclerosis Society of New Zealand. C.M. is a Travis Trust Senior Cancer Research Fellow. G.W.K was a recipient of a Wellcome Senior Research Fellowship in Medical Science in New Zealand and is currently a recipient of a James Cook Research Fellowship from the Royal Society of New Zealand. The initial EST clone used in this study was discovered as part of a joint collaboration between scientists at The Institute for Genomic Research (Rockville, MD), and at Human Genome Sciences Inc. (Rockville, MD).

REFERENCES

Adams, M. D., Kerlavage, A. R., Fleischmann, R. D., Fuldner, R. A., Bult, C. J., Lee N. H., *et al.* (1995). Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. *Nature* **377**(Suppl.): 3–174.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215:** 403–410.

Andersen, M. H., Berglund, L., Rasmussen, J. T., and Petersen, T. E. (1997). Bovine PAS-6/7 binds alpha v beta 5 integrins and anionic phospholipids through two domains. *Biochemistry* **36:** 5441–5446.

Appella, E., Weber, I. T., and Blasi, F. (1988). Structure and function of epidermal growth factor-like regions in proteins. *FEBS Lett.* **231:** 1–4.

Boyd, C. D., Toth-Fejel, S. E., Gadi, I. K., Litt, M., Condon, M. R., Kolbe, M., Hagen, I. K., Kurkinen, M., Mackenzie, J. W., and Magenis, E. (1988). The genes coding for human pro alpha 1(IV) collagen and pro alpha 2 (IV) collagen are both located at the end of the long arm of chromosome 13. *Am. J. Hum. Genet.* **42:** 309–314.

Campbell, I. D., and Bork, P. (1993). Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol.* **3:** 385–392.

Carrell, N. A., Fitzgerald, L. A., Steiner, B., Erickson, H. P., and Phillips, D. R. (1985). Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J. Biol. Chem.* **260:** 1743–1749.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.

Corson, G. M., Chalberg, S. C., Dietz, H. C., Charbonneau, N. L., and Sakai, L. Y. (1993). Fibrillin binds calcium and is coded by cDNAs that reveal a multidomain structure and alternatively spliced exons at the 5′ end. *Genomics* **17:** 476–484.

Dong, L-J., Hsieh, J-C., and Chung, A. E. (1995). Two distinct cell attachment sites in entactin are revealed by amino acid substitutions and deletion of the RGD sequence in the cysteine-rich epidermal growth factor repeat 2. *J. Biol. Chem.* **270:** 15838–15843.

Downing, A. K., Knott, V., Werner, J. M., Cardy, C. C., Campbell, I. D., and Handford, P. A. (1996). Solution structure of a pair of calcium-binding epidermal growth factor-like domains: Implications for the Marfan syndrome and other genetic disorders. *Cell* **85:** 597–605.

Engel, J. (1989). EGF-like domains in extracellular matrix proteins: Localized signals for growth and differentiation? *FEBS Lett.* **251:** 1–7.

Feng, G. S., Ouyang, Y. B., Hu, D. P., Shi, Z. Q., Gentz, R., and Ni, J. (1996). Grap is a novel SH3-SH2-SH3 adaptor protein that couples tyrosine kinases to the Ras pathway. *J. Biol. Chem.* **271:** 12129–12132.

Gilles, C., Piette, J., Ploton, D., Doco-Fenzy, M., and Foidart, J. M. (1996). Viral integration sites in human papilloma virus-33-immortalized cervical keratinocyte cell lines. *Cancer Genet. Cytogenet.* **90:** 63–69.

Graf, J., Iwamoto, Y., Sasaki, M., Martin, G. R., Kleinman, H. K., Robey, F. A., and Yamada, Y. (1987). Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell* **48:** 989–996.

Hynes, R. O. (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69:** 11–25.

Kanzaki, T., Olofsson, A., Morén, A., Wernstedt, C., Hellman, U. K., Claesson-Welsh, L., and Heldin, C.-H. (1990). TGF-beta 1 binding protein: A component of the large latent complex of TGF-beta 1 with multiple repeat sequences. *Cell* **61:** 1051–1061.

Kelsell, D. P., Rooke, L., Warne, D., Boucyk, M., Cullin, L., Cos, S., West, L., Povey, S., and Spurr, N. K. (1995). Development of a panel of monochromosomal somatic cell hybrids for rapid gene mapping. *Ann. Hum. Genet.* **59:** 233–241.

Knott, V., Downing, A. K., Cardy, C. M., and Handford, P. (1996). Calcium binding properties of an epidermal growth factor-like domain pair from human fibrillin-1. *J. Mol. Biol.* **255:** 22–27.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105–132.

Matsumura, K. (1995). Detection of DNA amplifications and deletions in oral squamous cell carcinoma cell lines by comparative

genomic hybridization (CGH). *Kokubyo Gakkai Zasshi J. Stomatol. Soc. Jpn.* **62:** 513–531.

Morén, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., Dijke, P., Miyazono, K., and Heldin, C. H. (1994). Identification and characterization of LTBP-2, a novel latent transforming growth factor-beta-binding protein. *J. Biol. Chem.* **269:** 32469–32478.

Morris, C. M., Courtay, C., Guerts van Kassel, A., ten Hoeve, J., Heisterkamp, N., and Groffen, J. (1993). Localization of a gamma-glutamyl-transferase-related gene family on chromosome 22. *Hum. Genet.* **91:** 31–36.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988). Electron microscopy and structural model of human fibronectin receptor. *EMBO J.* **7:** 4093–4099.

Panayotou, G., End, P., Aumailley, M., Timpl, R., and Engel, J. (1989). Domains of laminin with growth-factor activity. *Cell* **56:** 93–101.

Pereira, L., D'Alessio, M., Ramirez, F., Lynch, J. R., Sykes, B., Pangilinan, T., and Bonadio, J. (1993). Genomic organization of the sequence coding for fibrillin, the defective gene product in Marfan syndrome. *Hum. Mol. Genet.* **2:** 961–968.

Pikkarainen, T., Kallunki, T., and Tryggvason, K. (1988). Human laminin B2 chain. Comparison of the complete amino acid sequence with the B1 chain reveals variability in sequence homology between different structural domains. *J. Biol. Chem.* **263:** 6751–6758.

Ponten, J., and Saksela, E. (1967). Two established in vitro cell lines from human mesenchymal tumours. *Int. J. Cancer* **2:** 434–447.

Ramaswamy, H., and Hemler, M. E. (1990). Cloning, primary structure and properties of a novel human integrin beta subunit. *EMBO J.* **9:** 1561–1568.

Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G., and Stuart, D. (1995). The structure of a Ca(2+)-binding epidermal growth factor-like domain: Its role in protein–protein interactions. *Cell* **82:** 131–141.

Samec, S., Jones, T. A., Corlet, J., Scherly, D., Sheer, D., Wood, R. D., and Clarkson, S. G. (1994). The human gene for xeroderma pigmentosum complementation group G (XPG) maps to 13q33 by fluorescence *in situ* hybridization. *Genomics* **21:** 283–285.

Sheppard, D., Rozzo, C., Starr, L., Quaranta, V., Erle, D. J., and Pytela, R. (1990). Complete amino acid sequence of a novel integrin beta subunit (beta 6) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.* **265:** 11502–11507.

Takagi, J., Isobe, T., Takada, Y., and Saito, Y. (1997). Structural interlock between ligand-binding site and stalk-like region of β1 integrin revealed by a monoclonal antibody recognizing conformation-dependent epitope. *J. Biochem.* **121:** 914–921.

Taylor, M. R., Couto, J. R., Scallan, C. D., Ceriani, R. L., and Peterson, J. A. (1997). Lactadherin (formerly BA46), a membrane-associated glycoprotein expressed in human milk and breast carcinomas, promotes Arg-Gly-Asp (RGD)-dependent cell adhesion. *DNA Cell Biol.* **16:** 861–869.

Timpl, R., Aumailley, M., Gerl, M., Mann, K., Nurcombe, V., Edgar, D., and Deutzmann, R. (1990). Structure and function of the laminin–nidogen complex. *Ann. N.Y. Acad. Sci.* **580:** 311–323.

Tsuji, T., Okada, F., Yamaguchi, K., and Nakamura, T. (1990). Molecular cloning of the large subunit of transforming growth factor type beta masking protein and expression of the mRNA in various rat tissues. *Proc. Natl. Acad. Sci. USA* **87:** 8835–8839.

Wei, Y. F., Robins, P., Carter, K., Caldecott, K., Pappin, D. J., Yu, G. L., Wang, R. P., Shell, B. K., Nash, R. A., Schar, P., *et al.* (1995). Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination. *Mol. Cell Biol.* **15:** 3206–3216.

Yang-Feng, T. L., Li, S., Han, H., and Schwartz, P. E. (1992). Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. *Int. J. Cancer* **52:** 575–580.

Yin, W., Smiley, E., Germiller, J., Mecham, R. P., Florer, J. B., Wenstrup, R. J., and Bonadio, J. (1995). Isolation of a novel latent transforming growth factor-beta binding protein gene (LTBP-3). *J. Biol. Chem.* **270:** 10147–10160.

Yuan, Q., Jiang, W-M., Krissansen, G. W., and Watson, J. D. (1990). Cloning and sequence analysis of a novel β2-related integrin transcript from T lymphocytes: Homology of integrin cysteine-rich repeats to domain III of laminin B chains. *Int. Immunol.* **2:** 1097–1108.

Yuan, Q., Jiang, W-M., Leung, E., Hollander, D., Watson, J. D., and Krissansen, G. W. (1992). Molecular cloning of the mouse integrin beta 7 subunit. *J. Biol. Chem.* **267:** 7352–7358.

ELSEVIER

Biochimica et Biophysica Acta 1435 (1999) 61–70

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A family of *Arabidopsis* plasma membrane receptors presenting animal β-integrin domains

Valérie Laval [a], Matthieu Chabannes [a], Marguerite Carrière [a], Hervé Canut [a], Annick Barre [b], Pierre Rougé [b], Rafael Pont-Lezica [a], Jean-Philippe Galaud [a,*]

[a] *UMR CNRS/UPS 5546, Pôle de Biotechnologies Végétales, 24, Chemin de Borde-Rouge, BP 17, Auzeville, 31236 Castanet-Tolosan, France*

[b] *Institut de Pharmacologie et Biologie Structurale, UPR CNRS 9062, 205, route de Narbonne, 31077 Toulouse Cédex, France*

Received 16 June 1999; received in revised form 17 August 1999; accepted 18 August 1999

Abstract

A cDNA clone, *AtELP1* (*Arabidopsis thaliana* EGF receptor-like protein) was isolated from an *Arabidopsis* cDNA library with an oligonucleotide probe corresponding to a highly conserved region of animal β-integrins. The cloning of this cDNA was previously reported and it has been proposed that AtELP might be a receptor involved in intracellular trafficking. In the present work, using two specific independent sets of anti-peptide antibodies, we show that AtELP1 is mainly located in the plasma membrane, supporting another function for this protein. Structural studies, using methods for secondary structure prediction, indicated the presence of cysteine-rich domains specific to β-integrins. Database searches revealed that *AtELP1* is a member of a multigenic family composed of at least six members in *A. thaliana*. Northern blot analysis of *AtELP1*, *2b* and *3* was performed on mRNA extracted from cells cultured in normal and stressed conditions, and from several organs and plants submitted to biotic or abiotic stresses. All the genes are expressed at different levels in the same conditions, but preferentially in roots, fruits and leaves in response to water deficit.

Keywords: *Arabidopsis thaliana*; β-Integrin; Cysteine-rich domain; EGF domain; Plasma membrane receptor

1. Introduction

Plant cell morphogenesis is the result of numerous mechanisms involved in the control of cell division and expansion. The cell wall, the plasma membrane, and the cytoskeleton are considered as the main actors in the establishment of polarity and morphogenesis. It is now clear that the membrane and membrane proteins are kept in a dynamic state to maintain cell structure and compartmentalization [1]. Linkages between the plant plasma membrane and the cell wall can be observed after plasmolysis; however, the molecules engaged in this interaction are unknown. In animal cells, integrins are plasma membrane receptors involved in cellular adhesion. Some of them recognize extracellular proteins via the RGD sequence, a conserved motif in adhesion proteins from the extracellular matrix.

The occurrence of integrins in plants has been suggested, but their identification remains obscure. Two lines of evidence support the occurrence of integrin-like receptors in plants. On one hand, immunological cross reactivity between antibodies raised against animal integrins and plant proteins has been observed.

* Corresponding author. Fax: +33-562-193502; E-mail: galaud@cict.fr

0167-4838/99/$ – see front matter
PII: S0005-2728(99)00087-0

Immunological approaches identify several plant proteins sharing common epitopes with animal integrins [2–7]. Using an animal integrin polyclonal antibody for screening an *Arabidopsis* cDNA library, a membrane-associated protein involved in trafficking was isolated [8]. These results show that there are plant proteins sharing some motifs with animal integrins, but the proteins have no homology.

On the other hand, RGD peptides interfere with several plant physiological processes. Indeed, the addition of RGD peptides disrupts protoplast adhesion in tobacco derived from NaCl-adapted cells, [9]. These peptides also inhibit gravity perception in *Chara* [10], and enhance soybean cellular division [2]. Furthermore, in the brown alga *Fucus*, polarity determination is affected by the addition of RGD peptides [11]; and in *Uromyces*, a plant pathogenic fungus, appressorium formation is inhibit by the same compounds [5]. In agreement with these data, *Arabidopsis* plasma membrane exhibits specific high affinity binding sites for RGD-containing peptides and proteins. RGD binding is strongly inhibited by trypsin treatment, supporting the protein nature of the receptor [12].

In this paper, we use an oligonucleotide screening strategy to clone integrin-like molecules in *Arabidopsis*. The oligonucleotide probe is defined according to a cytoplasmic conserved region of integrin β-subunit, involved in interactions with the cytoskeleton [13]. If homology between animal and plant integrins exists, a functional domain involved in the interaction with cytoskeleton proteins will be present. Little homology is expected at the extracellular level since the extracellular matrices of animals and plants are completely different. The receptors binding to these matrices should reflect these differences [14].

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana, ecotype Columbia, was cultured in a grown chamber under fluorescent tubes 36 W (12 W/m^2) with 16-h light–8-h dark photoperiod. Plants were grown in pots filled with TKS2 peat Floratorf supplemented with 1‰ (w/w) nitrate. *Arabidopsis* cells were grown on Gamborg liquid medium [15]. Fifteen ml cell suspensions were routinely transferred to 300 ml fresh medium in 1000-ml Erlenmeyer flasks every 2 weeks, and shaken (150 rpm) in continuous light (60 W/m^2) at 26°C. Cells were transferred to fresh culture medium containing mannitol or not (250 mM), and maintained in the dark for different periods (1–15 days) prior to harvesting.

2.2. cDNA library screening, sequencing and computer sequences analysis

The *A. thaliana* cDNA library was constructed in pAD-GAL4 vector (Stratagene) and was kindly provided by B. Lescure (INRA-CNRS, Auzeville). A 17-mer degenerate oligonucleotide (AARTTYGAR-AARGARAA) corresponding to the peptide sequence KFEKEK was synthesized. This peptide matched a cytoplasmic conserved region from human integrin (β-subunit) [16]. The oligonucleotide was labeled with [γ-^{32}P]ATP by terminal transferase and used to screen the cDNA library according to Stratagene protocol. Positive clones were selected, excised from recombinant phage, and introduced into *Escherichia coli* strain SOLR. The isolated cDNAs were sequenced according to Sanger et al. [17].

The DNA and its deduced protein sequences were examined for homology in the non-redundant nucleotide and protein sequence databases using BLAST [18], PRODOM [19], and BLOCKS searches [20]. The amino acid sequence alignments were carried out on a Macintosh LC630 computer. The hydrophobicity, surface probability and flexibility profiles were calculated as described [21–23] with a window size of seven residues, using MacVector (Kodak). Hydrophobic cluster analysis (HCA) [24,25] was performed to delineate and compare the hydrophobic clusters along the amino acid sequences. They were generated on a Macintosh LC using the program HCA-Plot2 (Doriane, Paris, France).

2.3. RNA isolation and DNA–RNA hybridization analysis

Total RNA was extracted from *Arabidopsis* cell suspensions at various times during the culture and from different organs using the guanidinium thiocyanate method [26]. Total RNA (15 μg) was separated on formaldehyde agarose gel and blotted to Nytran

(Schleicher and Schuell) according to the manufacturer's specifications. The 3'-non-coding region of *AtELP1*, *2*, and *3* were amplified by PCR using degenerate primers, deduced from the highly conserved region of the three clones (5'-ATCATGKCACAG-TAYATGCCA-3') and a TTTTTTTTTTTTTTTW. Each PCR fragment was subcloned in pGEM-T vector (Promega), sequenced and used as specific probe.

2.4. Antibody production and purification

Specific antibodies raised against selected peptides derived from AtELP1 were prepared. Immunogenic peptides were defined by HCA and prediction of antigen determinants [27]. Two exposed hydrophilic regions, located between amino acids 352 and 365 (AEQESQIGKSRGDC, peptide 63), and amino acids 375–384 (NNRQYRGKLEC, peptide 64), were defined. BLAST analysis was carried-out to verify the presence of identical sequences in the *Arabidopsis* database. No other known protein, but the AtELP family, showed sequence 63 or 64 indicating that the chosen peptides could be specific for AtELP proteins. Both peptides were synthesized automatically by stepwise F-moc-*t*-butyl solid phase synthesis [28] in a Synergy Applied Biosystems peptide synthesizer. Crude synthetic peptides were purified by reverse-phase HPLC. Purified peptides were characterized by mass spectrometry on a Lasermat spectrometer (Finnigan), and coupled to the carrier protein. Peptides were coupled either to tyroglobulin or to bovine serum albumin using *N*-succimidyl-6-maleidocaproate as coupling reagent.

Before immunization, a sample of preimmune serum was taken and tested against peptides 63 and 64. In the absence of response, the immunization was performed. One volume of complete (immunization) Freund's adjuvant was added to the tyroglobulin-coupled peptide (250 μg per injection) and injected into rabbits. Two rabbits were immunized against each coupled peptide every 2 weeks during 3 months. Two antisera were obtained: serum 630 for peptide 63, and serum 640 for peptide 64. Antibodies were immunopurified before use. Ten micrograms of BSA-coupled peptide was separated by SDS-PAGE and transferred to nitrocellulose. The membrane was stained with Ponceau red. The stained region was cut, unstained, and blocked with TBS, 0.1% Tween, and 10% non-fat milk for 1 h at room temperature. The membrane was washed three times (15 min each) with TBS 0.1% Tween 1% BSA, and incubate overnight at 4°C with (1/25 dilution) serum. The antibodies were eluted with 500 μl glycine EGTA buffer (glycine 0.2 M, EGTA 1 mM, pH 2.8) and neutralized with 70 μl Tris 1 M pH 8.

2.5. Fractionation of A. thaliana membranes

Microsomes from *Arabidopsis* cells were prepared according to Bardy et al. [29] with a grinding medium containing 0.17 M sucrose, 50 mM KCl, 1 mM DTT, and 10 mM HEPES, pH 7.5. Microsomes were separated by free-flow electrophoresis with an Elphor Vap-22 electrophoresis unit (Weber, Kirchheim-Heimstetten, Germany). The electrophoresis medium contained 0.25 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 10 mM Tris and 10 mM boric acid (pH 8.3). The electrode buffer consisted of 100 mM Tris, 100 mM boric acid (pH 8.3). Microsomes were resuspended in electrophoresis medium and centrifuged for 30 min at $45\,000 \times g$. Electrophoresis was performed at a 100 mA constant current (about 900 V), sample injection 2 ml h^{-1}, and buffer flow 3.5 ml $fraction^{-1}$ h^{-1} at 4°C. The distribution of membranes in each separation was monitored by absorbance at 280 nm. Membranes were collected from pooled fractions by centrifugation (30 min at $45\,000 \times g$). Activity of different marker enzymes was determined as previously described [29]. Protein content was determined as reported [30] with bovine serum albumin as standard.

2.6. Gel electrophoresis and immunodetection

Gel electrophoresis was carried-out on 11% acrylamide gels. Samples (50 μg purified protein) were solubilized in 0.125 M Tris pH 6.8, 4% SDS and 20% glycerol prior to electrophoresis. Proteins were transferred to nitrocellulose, and incubated overnight with 630 or 640 (1/100 dilution) purified primary antibodies, washed, and revealed with ImmunoPure ABC phosphatase staining kit (Pierce).

Antibody competition was realized by incubation of 1 mg non-coupled peptide with its corresponding antibody for 2 h at 37°C. The exhausted antibody

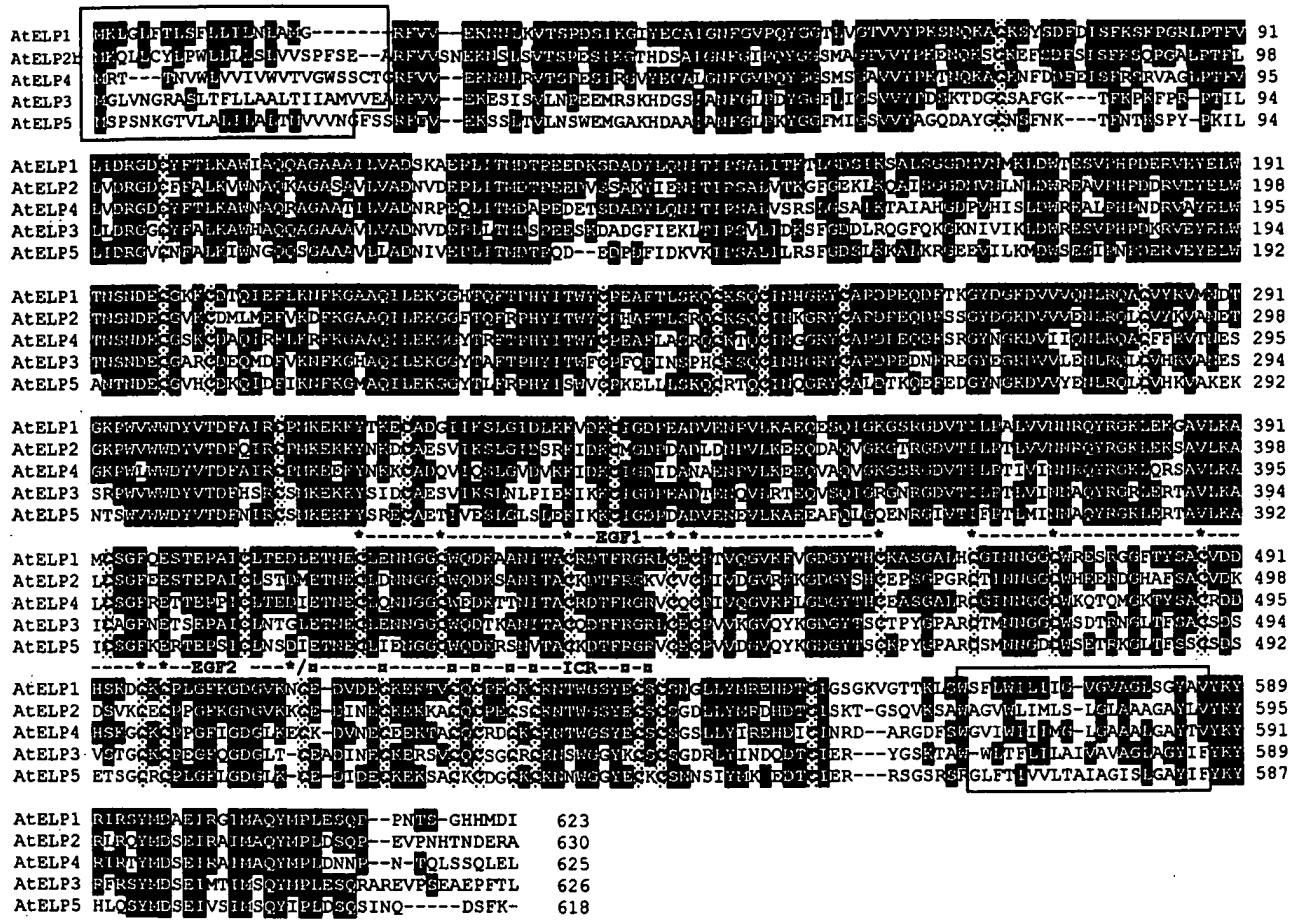


Fig. 1. Comparison of the predicted protein sequences of AtELP1, 2b, 3, 4, and 5. Residues identical in all the proteins are highlighted and the conserved cysteine residues are in gray. EGF signature (epidermal growth factor) is shown by * and ICR (integrin cysteine-rich motif) by a boxed circle. Putative peptide signal and transmembrane domains are boxed. Spaces, denoted by dashes have been introduced to optimize the alignment.

was then incubated with the nitrocellulose membranes.

3. Results

3.1. Molecular cloning, homology searches, and protein sequence analysis

An *A. thaliana* cDNA library was screened with an oligonucleotide probe corresponding to a conserved cytoplasmic region of integrin β-subunits. Seven clones were isolated and partially sequenced. One clone (2712), presenting a potential transmembrane domain, was completely sequenced. The cDNA insert was found to be 2314 bp in length. It encodes a complete 623 amino acid protein, which has a predicted molecular mass of 70 kDa and a potential membrane-spanning domain. Database search revealed that this clone was independently identified by three other groups at the same time [31]. Clone *2712* will be called *AtELP1* (*A. thaliana* EGF-like protein) in this paper.

Southern blot experiments using *AtELP1* cDNA as a probe (data not shown) revealed that *AtELP1* belongs to a multigenic family. Homology searches in *A. thaliana* EST database showed two nucleic acid sequences having 81 and 63% homology with *AtELP1*. These sequences were called, respectively, *AtELP2* (accession number U79960) and *AtELP3* (EST accession number 110G6T7). The complete genomic sequence of *AtELP3* (accession number

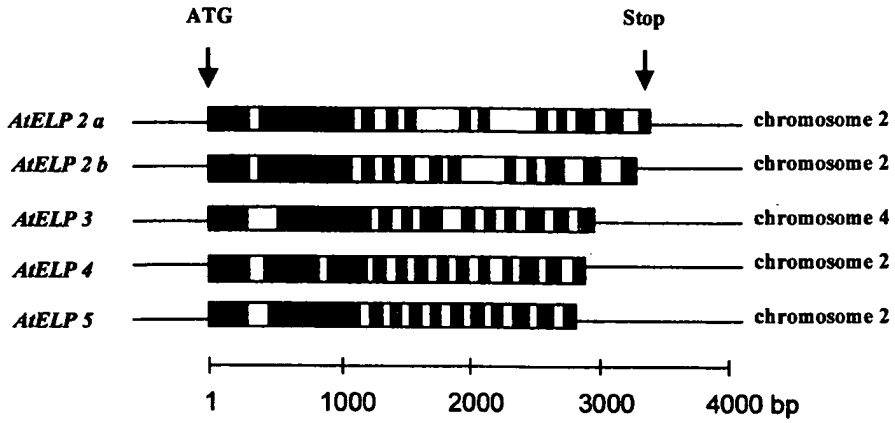


Fig. 2. Schematic representation of *AtELP2a*, *2b*, *3*, *4*, and *5* genomic clones. *AtELP2a*, *2b* (BAC F26C24), *4* and *5* (BAC T09D09, and BAC F19I3) are located on chromosome 2. *AtELP3* (BAC F18F4) is located on chromosome 3. Exons are represented by gray boxes and introns by white boxes. ATG represent the translation start codon.

AL021637, pid g 2827665) and two other clones presenting homologies with *AtELP1* were deduced from the analysis of the *A. thaliana* genomic database. These clones were named *AtELP4* (accession number ATAC002338, pid g 2347209) and *AtELP5* (accession number ATAC004238, pid g 3033390) and their nucleic acid sequences showed 84 and 61% homology with *AtELP1*.

Primary and secondary sequence analyses were performed on AtELP1. The polypeptide chain is rather hydrophilic (64% polar residues) and contains a high proportion of cysteine (34 residues). Its calculated isoelectric point is 5.87. The polypeptide shares moderate percentages of identity (14.8 and 17.8%) and homology (34.6 and 38.8%) with human β1 and β5 integrins.

The deduced protein sequences of the five clones are shown in Fig. 1. AtELP proteins ranged from 618 to 630 amino acids. They present the same common structural features: a potential signal peptide at the N-terminus, a large N-terminal region, a potential transmembrane domain, and a short C-terminal region. Alignment of AtELP1, 2, and 4 showed about 80% homology. The N-terminus contains many conserved regions. The number and position of cysteines is well conserved (34 Cys out of 560 amino acids). Three Cys-rich motifs were found in all the putative proteins. Two of them (EGF1 and EGF2, in Fig. 1) have the typical arrangement $Cx_{(3-7)}Cx_{(2-6)}Cx_{(7-10)}CxCx_{(7-12)}C$ common to epidermal growth factors (EGF) with 6 cysteines in conserved positions. The third Cys-rich motif has a different organization with eight cysteines in the following sequence: $Cx_{(6-8)}Cx_5CxCxxCxCx_{(8-13)}Cx_{(1-2)}C$. This cysteine alignment is characteristic of β-integrin subunits (ICR, integrin Cys-rich motif in Fig. 1). The C-terminal domain of AtELPs (34–40 amino acids) contains a highly conserved sequence of 27 amino acids, but then diverges. It contains a YMPL site (amino acid 606–609). The Yxxϕ motif (x represents any amino acid and ϕ a hydrophobic residue) has been demonstrated to mediate internalization from the cell surface as well as targeting to intracellular compartments in mammals [32]. The more divergent sequences correspond to the potential signal peptide, the putative transmembrane domain, and the C-terminus end.

3.2. Structure of genomic clones

The structure of five *AtELP* genes is presented in Fig. 2. These genomic sequences were obtained by the systematic sequencing programs [33]. Two sequences, *AtELP2a* (accession number ATAC 004705, pid 3252813) and *AtELP2b* (accession number ATAC 004705, pid 3252815, which corresponds to the cDNA previously described as *AtELP2*), encode proteins showing 96.5% identity (22 different amino acids out of 628). The length of the ORF is the same for both genes, but they have different intron lengths. *AtELP2a* and *AtELP2b* are located on

chromosome 2 in reversed position, and are separated by a single gene. *AtELP2a* and *2b* could be the result of gene duplication. *AtELP4* (accession number ATAC 002338, pid 2347209) and *AtELP5* (accession number ATAC 004238, pid 3033390) are

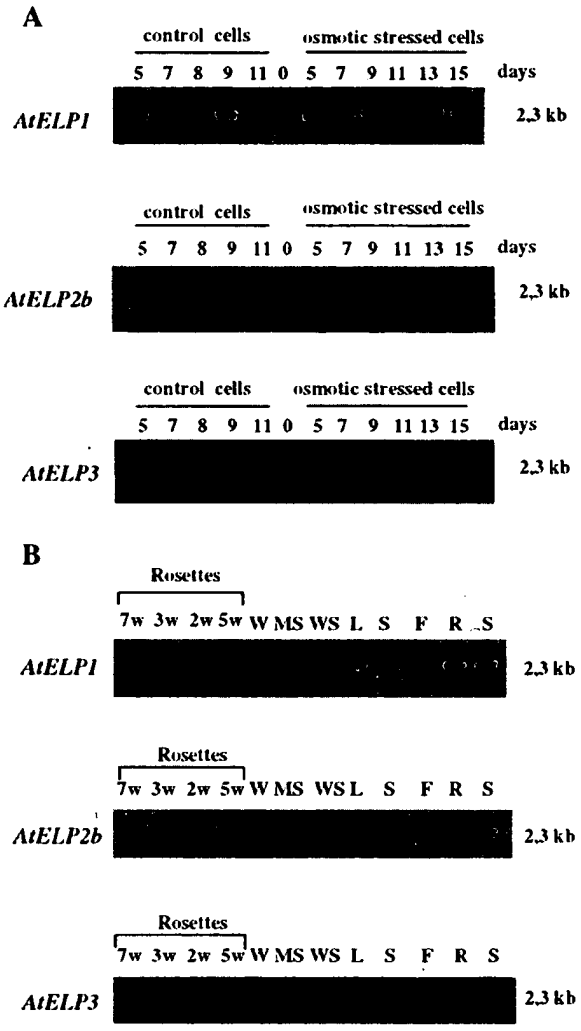


Fig. 3. (A) Northern blot analysis of *AtELP1, 2b*, and *3* gene expression during cell culture in control and osmotic stress conditions. Total RNA was extracted from cells cultured with 250 mM mannitol (osmotic stress) or not (control cells). Total RNA (15 μg) was separated on formaldehyde gel blotted and probed with ^{32}P-specific 3′-UTR from each clone. (B) Northern blot analysis of *AtELP1, 2b*, and *3* gene expression in various organs of *Arabidopsis*. Total RNA was extracted from: leaves (L), stem (S), flowers (F), roots (R), siliques (S) and rosettes at different developmental stages (7w, 7 weeks; 3w, 3 weeks; 2w, 2 weeks; 5w, 5 weeks) and after several stresses (W, wounding; MS, mechanical stress; WS, water stress).

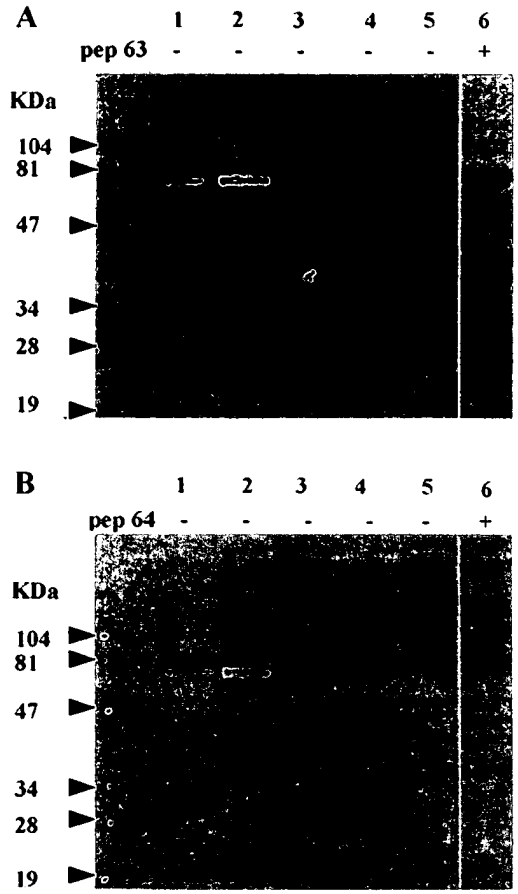


Fig. 4. Western blot analysis of AtELP1 on purified membrane fractions by free-flow electrophoresis. Membranes were prepared from cells cultured in mannitol containing medium. Fractions number 1 and 2 correspond to plasma membrane, fractions 3 and 4 to endomembrane (Golgi, ER, mitochondria...) and fraction 5 to tonoplast. Lane 6 corresponds to plasmalemma (lane 2). Fifty μg protein were separated on by 11% SDS PAGE gel, blotted onto nitrocellulose and incubated with antibody 630 (A) and antibody 640 (B) in the presence (+) or in absence (−) of the corresponding peptide. Molecular mass standards are given on the left of the figure.

also located on chromosome 2. The genomic sequence corresponding to *AtELP3* (accession number ALO21637, pid 2827665) is the only one located on chromosome 4. At present, the *AtELP1* genomic clone has not been found. The multigenic family *AtELP* is composed of at least six genes having 11–13 exons and 10–12 introns. Among these six genes, only three have so far been found expressed (*AtELP1*, *AtELP2b*, and *AtELP3*).

Sequence analysis of each promoter was carried out using the Transfac program [34]. No clearly iden-

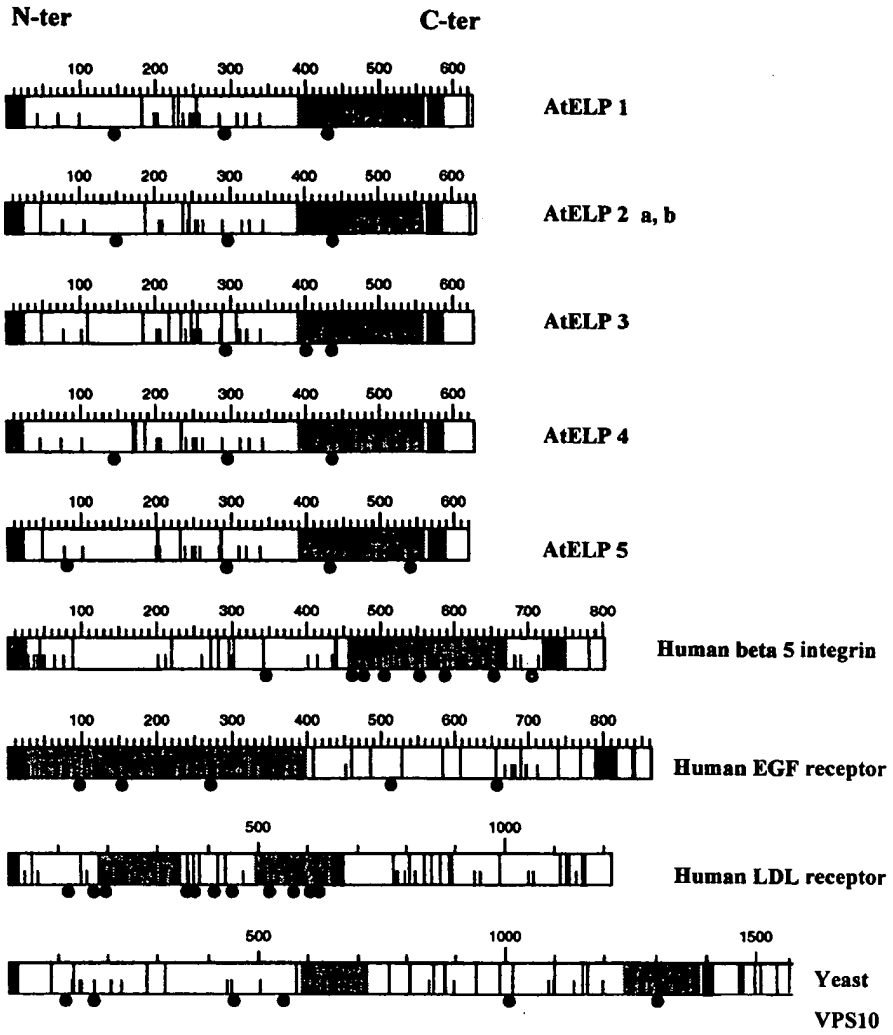


Fig. 5. Schematic comparison of AtELP1, 2a, 2b, 3, 4, 5 with other cysteine-rich proteins. Human β5-integrin subunit (accession number M35011), human EGF receptor (accession number X00588), human LDL receptor (accession number L00352) and the yeast VPS10 (accession number U07621). Cysteine-rich domains are represented by gray boxes and cysteines by small bars. At the N-ter end, peptide signal is represented by hatched boxes; transmembrane domains are black boxed and black circles represent putative glycosylation sites.

tifiable regions corresponding to putative *cis*-acting elements were found in *AtELP* promoter genes.

3.3. *AtELP1, AtELP2b and AtELP3 gene expression analysis*

Analysis of *AtELP1* gene expression was done using the 3′ non-coding region, cloned after PCR amplification. Total RNA was extracted from dark cultured cells under control or osmotic stress (Fig. 3A). A single signal corresponding to a 2.3-kb transcript was observed. This signal was increased during the culture period under osmotic stress, compared to control cells. In Fig. 3B, *AtELP1* gene expression analysis was performed on total RNA extracted from different organs or after various stress: mechanical, wounding, water deficit, and after *Ralstonia solanacearum* infection (data not shown). A weak signal was observed in young plantlets (rosette stage) compared to other organs (leaf, root, and stem). Stress was applied on plantlets at 5 weeks rosette stage, and the higher signal was observed in plants

left without water for 24 h. *AtELP2b* and *AtELP3* gene expression was analyzed using specific probes, 3′-non-coding region were specifically PCR amplified before use. Expression of *AtELP2b* is weakly enhanced compared to *AtELP1* (Fig. 3A,B) and the basal expression level of *AtELP3* is weaker than the other genes under the conditions used.

3.4. AtELP1 localization

Two sets of antibodies (630 and 640) raised against peptides derived from the AtELP1 primary sequences were used for the localization of the protein. Purified membranes were obtained and characterized as described [29]. The proteins were separated on SDS gel electrophoresis, transferred to nitrocellulose membranes, and revealed using antibody 630. A single band around 80 kDa was observed in plasma membrane and endomembrane enriched fractions (Fig. 4A). The higher signal was observed in fraction 2 (plasma membrane). This signal strongly decreased when antibody 630 was pre-incubated with its corresponding peptide (lane 6). The same results were obtained when antibody 640 was used (Fig. 4B).

4. Discussion

A new class of membrane proteins (AtELPs) has been identified. Database search revealed that *AtELP1* was simultaneously cloned by three other groups. Whereas the primary sequence of the protein does not show obvious homology with known proteins from plants or other organisms, it has been proposed that AtELP might be a receptor involved in plant intracellular protein trafficking [35,36]. AtELP1's secondary structure was compared with that of two well-known trafficking sorting proteins: the yeast VSP10 and the rat mannose-6-phosphate receptor (M6PR). Dot-plots performed with the PAM250 matrix [37] showed a limited number of very short diagonals. This indicated that no consistent similarities occurred between the proteins (data not shown). In addition, the Cys-rich domains located close to the C-terminal of AtELP1 do not occur in other proteins.

HCA analysis of AtELP1 and human integrins β1 and β5 reinforced the structural comparison indicating that these three proteins exhibit a very similar molecular organization characterized by the Cys-rich domains. AtELP1 and β-integrin subunits have 13.1 and 15% cysteine, respectively, on the stretch preceding the transmembrane domain [38]. These cysteines are presumably disulfide-bonded and such bonding would necessarily occur in the extracellular domain [39]. As in the β-integrin family, AtELPs contain two EGF-like signatures. The third Cys-rich domain present in AtELPs is characteristic of β-integrins as indicate in the PROSITE database. Such Cys-rich repeats seem to stabilize the integrin structure at the base of the protein.

EGF domains have been found in a large number of proteins and their common feature is to be present in the extracellular domain of membrane or secreted proteins, with the exception of a prostaglandin G/H synthase. In SWISS-PROT database, 49 proteins present the EGF signature, containing six cysteines. In the mammalian EGF and LDL receptors, EGF regions seem to be involved in receptor–ligand interactions at the cell surface of animal cells [40,41].

Fig. 5 compares AtELP1–5 with other Cys-rich membrane proteins, like the LDL receptor, the EGF receptor, the yeast Vps10, and the human integrin β5. From a structural point of view, AtELPs seem to be closer to the integrin family than to the endocytic or sorting receptors. AtELPs and the β-subunit of animal integrins may be derived from a common ancestor. AtELPs may be considered as integrin orthologous proteins. Brower et al. [42] suggest that cell surface receptors are strongly conserved in higher animals, but their molecular evolution remains obscure. The cloning of two cDNAs encoding integrin β-subunits from coral and sponge clearly showed that the major structural features were well conserved. Comparative analysis of the genome of the nematode *Caenorhabditis elegans* showed that the main differences observed between the two genomes corresponded to proteins involved in the establishment of multicellularity (adhesion molecules) and cell death machinery (signaling proteins) [43]. Brower et al. [42] indicated that even if no protein with obvious homology to integrins has been identified, the existence of integrin-like molecules in plants [4,12] and in fungi [44,45] has been reported by several authors.

Integrins recognize the RGD sequence in their li-

gand via interaction with DxSxS, an integrin binding motif [46]. The presence of an equivalent motif is observed in AtELP1, 2a, and 2b proteins at the N-terminal domain. The presence on the same polypeptide of a motif binding RGD (DxSxS), and an exposed RGD sequence is puzzling. A RGD sequence in the N-terminus is also present in the extracellular domain of human β2, β5 and β6 integrins. Papadopoulos et al. [47] reported that 7182 proteins contain the RGD motif. Of these proteins, only 120 are membrane or membrane-associated proteins having the RGD sequence in their extracellular domain and some are proteins involved in cell adhesion processes.

Ahmed et al. [36] showed that the C-terminal region of AtELP1 is located in the cytosol. The cytoplasmic domain of AtELPs is well conserved and it contains the Yxxϕ motif at a distance of about 20 amino acids from the membrane-spanning region. This sequence is a recognition motif for endocytosis through clathrin-coated vesicles [32]. In animal integrins, the presence of the signal sequence (NPxY), localized about 20–25 amino acids from the transmembrane domain [16], is required for internalization via clathrin-coated vesicles [48]. In this line, the vitronectin receptor αvβ5 plays a double role in fibroblasts; it binds to and directly internalizes vitronectin. The presence of AtELP on the plasma membrane indicates that the protein follows the secretory pathway when it is synthesized, and may be internalized via clathrin-coated vesicles into the vacuolar compartment. This is supported by the presence of the protein in the trafficking vesicles [35], as well as for the presence of internalization signals in the cytoplasmic tail.

At present, six genes encoding AtELP were identified in *Arabidopsis*, but only three were expressed, referred to EST and cDNA sequencing programs. The main accumulation of transcripts was observed in roots, in cultured cells and in young plantlets submitted to water stress. Previous work from Katembe et al. [6], showed that integrin-like molecules were accumulated in *Arabidopsis* roots, an important gravity perception site. Wayne et al. [10] reported that gravisensing of *Chara* cells was dependent on RGD binding and a collagenase treatment indicated the presence of a PxGP motif in the RGD gravireceptor sequence. This motif is present in a conserved position in AtELP2a, 2b, 3, and 5 in the large N-terminal region, but the biological significance is unknown.

Acknowledgements

V.L. is a fellow from the Ministere de l'Education Nationale, de la Recherche et de la Technologie, France. We are especially grateful to N. Vita (Sanofi-Recherche, Labège) and H. Mazarguil (IPBS, Toulouse) for their helpful collaboration, and to the *Arabidopsis* Biological resource Center, Ohio State University, for providing EST clones. 'Merci à Lorena' for English revision. This project was supported by Université Paul Sabatier and by the Centre National de la Recherche Scientifique (Grants 96N88/0010 and 285016).

References

[1] J.E. Fowler, R.S. Quatrano, Annu. Rev. Cell Dev. Biol. 13 (1997) 697–743.
[2] M. Schindler, S. Meiners, D.A. Cheresh, J. Cell Biol. 108 (1989) 1955–1965.
[3] R.S. Quatrano, L. Brian, J. Aldridge, T. Schultz, Development 1 (Sup.) (1991) 11–16.
[4] J.S. Gens, C. Reuzeau, K. Doolittle, J.G. McNally, B.G. Pickard, Protoplasma 194 (1996) 215–230.
[5] A. Corrêa, R.C. Staples, H.C. Hoch, Protoplasma 194 (1996) 91–102.
[6] W.J. Katembe, L.J. Swatzell, C.A. Makaroff, J.K. Kiss, Physiol. Plant. 99 (1997) 7–14.
[7] A. Faik, A.M. Labouré, D. Gulino, P. Mandaron, D. Falconet, Eur. J. Biochem. 253 (1998) 552–559.
[8] J.P. Galaud, V. Laval, M. Carriere, A. Barre, H. Canut, P. Rouge, R. Pont-Lezica, Biochim. Biophys. Acta 1341 (1997) 79–86.
[9] J.K. Zhu, J. Shi, U. Singh, S.E. Wyatt, R.A. Bressan, P.M. Hasegawa, N.C. Carpita, Plant J. 3 (1993) 637–646.
[10] R. Wayne, M.P. Staves, A.C. Leopold, J. Cell Sci. 101 (1992) 611–623.
[11] D.L. Kropf, B. Kloareg, R.S. Quatrano, Science 239 (1988) 187–190.
[12] H. Canut, A. Carrasco, J.P. Galaud, C. Cassan, H. Bouyssou, N. Vita, P. Ferrara, R. Pont-Lezica, Plant J. 16 (1998) 63–71.
[13] M. Pfaff, S. Liu, D.J. Erle, M.H. Ginsberg, J. Biol. Chem. 273 (1998) 6104–6109.
[14] C. Reuzeau, R.F. Pont-Lezica, Protoplasma 152 (1995) 113–121.

[15] M. Axelos, C. Curie, L. Mazzolini, C. Bardet, B. Lescure, Plant Physiol. Biochem. 30 (1992) 123–128.
[16] S. K Sastry, A.F. Horwitz, Curr. Opin. Cell Biol. 5 (1993) 819–831.
[17] F. Sanger, S. Nicklens, A.R. Coulson, Proc. Natl. Acad. Sci. USA 74 (1997) 5463–5467.
[18] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D. Lipman, J. Mol. Biol. 215 (1990) 403–410.
[19] E.L. Sonnhammer, D. Kahn, Protein Sci. 3 (1994) 482–492.
[20] S. Henikoff, J.G. Henikoff, Genomics 19 (1994) 97–107.
[21] J. Kyte, R.F. Doolittle, J. Mol. Biol. 157 (1982) 105–132.
[22] J. Janin, Nature 277 (1979) 491–492.
[23] P.A. Karplus, G.E. Schulz, Naturwissenschaften 72 (1985) 212.
[24] C. Gaboriaud, V. Bissery, T. Benchetrit, J.P. Mornon, FEBS Lett. 224 (1987) 149–155.
[25] L. Lemesle-Varloot, B. Henrissat, C. Gaboriaud, V. Bissery, A. Morgat, J.P. Mornon, Biochimie 72 (1990) 555–574.
[26] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[27] T.P. Hopp, K.R. Woods, Proc. Natl. Acad. Sci. USA 78 (1981) 3824–3828.
[28] G.B. Fields, R.L. Noble, Int. J. Pept. Protein Res. 35 (1990) 161–214.
[29] N. Bardy, A. Carrasco, J.P. Galaud, R. Pont-Lezica, H. Canut, Electrophoresis 19 (1998) 1145–1153.
[30] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[31] J.-M. Neuhaus, J.C. Rogers, Plant Mol. Biol. 38 (1998) 127–144.
[32] H. Ohno, J. Stewart, M.-C. Fournier, H. Bosshart, I. Ree, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, J.S. Bonifaciono, Science 269 (1995) 1872–1875.
[33] M.L. Wang, L. Huang, D.K. Bongard-Pierce, S. Belmonte, E.A. Zachgo, J.W. Morris, M. Dolan, H.M. Goodman, Plant J. 12 (1997) 711–730.
[34] T. Heinemeyer, E. Wingender, I. Reuter, H. Hermjakob, A.E. Kel, O.V. Kel, E.V. Ignatieva, E.A. Ananko, O.A. Podkolodnaya, F.A. Kolpakov, N.L. Podkolodny, N.A. Kolchanov, Nucleic Acids Res. 26 (1998) 362–367.
[35] N. Paris, S.W. Rogers, L. Jian, T. Kirsch, L. Beevers, T.E. Phillips, J.C. Rogers, Plant Physiol. 115 (1997) 29–39.
[36] S.V. Ahmed, M. Bar-Peled, N.V. Raikhel, Plant Physiol. 114 (1997) 325–336.
[37] M.O. Dayhoff, R.V. ECK, C.M. Park, in: Atlas of Protein Sequence and Structure, Vol. 5, Natl. Biomed. Foundation, Washington, DC, USA, 1972.
[38] J.W. Tamkun, D.W. DeSimon, D. Fonda, R.S. Patel, C. Buck, A.F. Horwitz, R.O. Hynes, Cell 46 (1986) 271–282.
[39] J.J. Calvete, A. Henschen, J. Gonzalez-Rodriguez, Biochem. J. 274 (1991) 63–171.
[40] T. Yamamoto, C.G. Davis, M.S. Brown, W.J. Schneider, M.L. Casey, J.L. Goldstein, D.W. Russell, Cell 39 (1984) 27–38.
[41] A. Lalazar, K.H. Weisgraber, S.C. Ra, H. Giladi, T.K.L. Inneraarity, A.Z. Levanon, J.K. Boyles, B. Amit, M. Gorecki, R.W. Mahley, J. Biol. Chem. 263 (1988) 3542–3545.
[42] D.L. Brower, S.M. Brower, D.C. Hayward, E.E. Ball, Proc. Natl. Acad. Sci. USA 94 (1997) 9182–9187.
[43] S.A. Chervitz, L. Aravind, G. Sherlock, C.A. Ball, E.V. Koonin, S.S. Dwight, M.A. Harris, K. Dolinski, S. Mohr, T. Smith, S. Weng, J.M. Cherry, D. Botstein, Science 282 (1998) 2022–2028.
[44] C. Gale, D. Finkel, N. Tao, M. Meinke, M. McClellan, J. Olson, K. Kendrick, M. Hostetter, Proc. Natl. Acad. Sci. USA 93 (1996) 357–361.
[45] C.A. Gale, C.M. Bendel, M. McClellan, M. Hauser, J.M. Becker, J. Berman, M.K. Hostetter, Science 279 (1998) 1355–1358.
[46] M.L. Bajt, J.C. Loftus, J. Biol. Chem. 269 (1994) 20913–20919.
[47] G.K. Papadopoulos, C. Ouzoumis, E. Eliopoulos, Int. J. Biol. Macromol. 22 (1998) 51–57.
[48] W.J. Chen, J.L. Goldstein, M.S. Browns, J. Biol. Chem. 265 (1990) 3116–3123.